Characterization of the *Sucrose Synthase* and class II *Trehalose 6-Phosphate Synthase gene* families in the moss *Physcomitrella patens*.

by

Anna Johanna Wiese

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Supervisor: Prof. James Richard Lloyd

Faculty of Agrisciences

Department of Genetics

Institute for Plant Biotechnology

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Declaration

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December 2020

Abstract

Plant sugars have dual functionality, in that they play a role in primary metabolism and partake in signal transduction pathways. As it relates to their signaling function, they relay information to the nucleus regarding energy status, allowing the plant to adapt accordingly. Most of what is known about these functions of sugars have come from research in vascular plants, leaving aspects thereof unaddressed in non-vascular plants. Certain bryophytes (non-vascular plants) have been developed over into model plants, with ***Physcomitrella patens*** representing one such model. This plant has become popular in studies of evolutionary development and non-vascular plant biology. In this study, I characterized two gene families in *P. patens*, namely **sucrose synthases** (**SUS**) and **trehalose 6-phosphate synthases** (**TPS**), whose homologs in higher plants are implicated in sugar metabolism and signaling.

Sucrose, the end-product of photosynthesis, is central to primary carbon metabolism. Its synthesis and degradation are tightly controlled to balance out supply and demand throughout the plant. Sucrose synthases are implicated in phloem loading and sink strength in vascular plants, where its cleavage products can enter primary metabolism or be used in the synthesis of complex carbohydrates. Little is known about SUS function in non-vascular plants, and in this study, I report the characterization of four putative SUS homologs in *P. patens*. Phylogenetic classification of land plant *SUS* sequences revealed the existence of 5 clades, one of which contained only bryophyte-sequences including all those from *P. patens*. Analysis of the amino acid sequences revealed that residues involved in SUS regulation in higher plants were conserved in PpSUS proteins. I was able to demonstrate SUS activity in crude protein extracts, however, detailed kinetic characterization was hindered by protein expression in *E. coli*. Localization studies revealed that all PpSUS proteins were cytosolic, while expression analyses indicated that *PpSUS* genes have overlapping and unique expression patterns.

Another sugar which is implicated in signaling is trehalose 6-phosphate (Tre6P), an intermediate in the trehalose biosynthesis pathway. Levels of this sugar phosphate change in parallel to that of sucrose, with researchers proposing that it plays a role in communicating sucrose availability. The second part of this study focussed on the proteins involved in Tre6P synthesis, namely trehalose 6-phosphate synthases (TPSs), which are divided into two classes, with class I proteins containing catalytically active polypeptides (Leyman et al., 2001; Lunn, 2007). Very little is known about the class II proteins, and in this study, I characterized members of this class in *P. patens*. *Physcomitrella* contains six *TPS* genes in its genome, four

of which encode class II proteins. Phylogenetic classification differentiated TPS sequences from land plants into 2 clades (I and II) consisting of 7 sub-clades (IA-B and IIA-E), suggesting the existence of 1 ancestral *TPS* gene. Functional complementation revealed weak TPS catalytic activity for one of the class II TPS proteins, a first for any plant class II protein studied to date. Subcellular localization experiments conducted on three of the class II proteins revealed that they were cytosolic, while yeast two hybrid analyses indicated that these proteins do not form complexes with each other or the class I proteins. Finally, expression analyses indicated that class II genes have overlapping expression patterns. This study provides novel insights into the evolution of *SUS* and *TPS* genes in *P. patens* and, will serve as a platform for the design of future experiments related to these gene families in non-vascular plants.

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Opsomming

Plantsuikers het tweeledige funksionering deurdat dit 'n rol speel in primêre metabolisme en deelneem aan seintransduksieweë. Met betrekking tot hul seinfunksie, stuur suikers inligting oor die energiestatus na die kern, wat die plant in staat stel om aanpassings te maak. Meeste van wat ons weet oor hierdie funksies van suikers, kom uit navorsing in vaatplante, wat aspekte daarvan in nie-vaatplante onbekend laat. Sekere bryofiete (nie-vaatplante) is ontwikkel tot modelplante, waarvan *Physcomitrella patens* een sulke model verteenwoordig. Hierdie plant het gewild geword in studies oor evolusionêre ontwikkeling en nie-vaskulêre plantbiologie. In hierdie studie het ek twee geenfamilies in ***P. patens*** gekenmerk, naamlik **sukrose sintases** (**SUS**) en **trehalose 6-fosfaat sintases** (**TPS**), die homoloë waarvan in hoër plante betrokke is by suikermetabolisme en seine.

Sukrose, die eindproduk van fotosintese, is sentraal tot die primêre koolstof metabolisme. Die sintese en afbraak daarvan word streng beheer om die vraag en aanbod in die hele plant te balanseer. Sukrose sintases word geïmpliseer in floeem lading en sink sterkte in vaatplante, waar die afbreek produkte daarvan primêre metabolisme kan binnegaan of in die sintese van komplekse koolhidrate gebruik kan word. Baie min is bekend oor SUS funksie in nie-vaatplante, en in hierdie studie rapporteer ek die karakterisering van vier vermeende SUS homoloë in *P. patens*. Filogenetiese klassifikasie van *SUS* sekwense van landplante het aan die lig gebring dat daar vyf klades bestaan, waarvan een slegs bryofiet-sekwense bevat, insluitend almal van *P.patens*. Analise van die aminosuur volgordes het aan die lig gebring dat residue wat by SUS regulering by hoër plante betrokke was, ook in PpSUS proteïene gekonserveer is. Ons kon SUS aktiwiteit in ruwe proteïen ekstrakte demonstreer, maar gedetailleerde kinetiese karakterisering is verhinder deur proteïen uitdrukking in *E. coli*. Lokalisasie studies het aan die lig gebring dat alle PpSUS proteïene sitosolies was, terwyl uitdrukkings analises aangedui het dat *PpSUS* gene oorvleuelende en unieke uitdrukkings patrone het.

'N Ander suiker wat by seintransduksie geïmplementeer word, is trehalose 6-fosfaat (Tre6P), 'n tussenproduk in die trehalose biosintese-weg. Die vlakke van hierdie suikerfosfaat verander in parallel met die van sukrose, en navorsers stel voor dat dit 'n rol speel in die kommunikasie van die beskikbaarheid van sukrose. Die tweede deel van hierdie studie het gefokus op die proteïene wat betrokke is by Tre6P-sintese, naamlik trehalose 6-fosfaat sintases (TPS's), wat in twee klasse verdeel is, met klas I proteïene wat katalities aktiewe proteïene bevat (Leyman et al., 2001; Lunn, 2007). Daar is baie min bekend oor die klas II-proteïene, en in hierdie studie

het ek lede van hierdie klas in *P. patens* gekenmerk. *Physcomitrella* bevat ses *TPS* gene in sy genoom, waarvan vier vir klas II proteïene kodeer. Filogenetiese indeling het TPS sekwense van landplante in 2 klades (I en II) gedifferensieer, bestaande uit 7 subklaaie (IA-B en IIA-E), wat daarop dui dat daar 1 voorouerlike *TPS* geen bestaan. Funksionele komplementering het swak TPS katalitiese aktiwiteit getoon vir een van die klas II TPS proteïene, ń eerste vir enige plant klas II proteïen wat tot dusver bestudeer is. Sub-sellulêre lokaliserings eksperimente wat op drie van die klas II proteïene uitgevoer is, het aan die lig gebring dat hulle sitosolies was, terwyl interaksie analises aangedui het dat hierdie proteïene nie komplekse met mekaar of die klas I proteïene vorm nie. Laastens het uitdrukkings analises aangedui dat klasse II gene oorvleuelende uitdrukkings patrone het. Hierdie studie bied nuwe insigte in die evolusie van *SUS* en *TPS* gene in *P. patens* en sal dien as 'n platform vir die ontwerp van toekomstige eksperimente wat verband hou met hierdie geenfamilies in nie-vaatplante.

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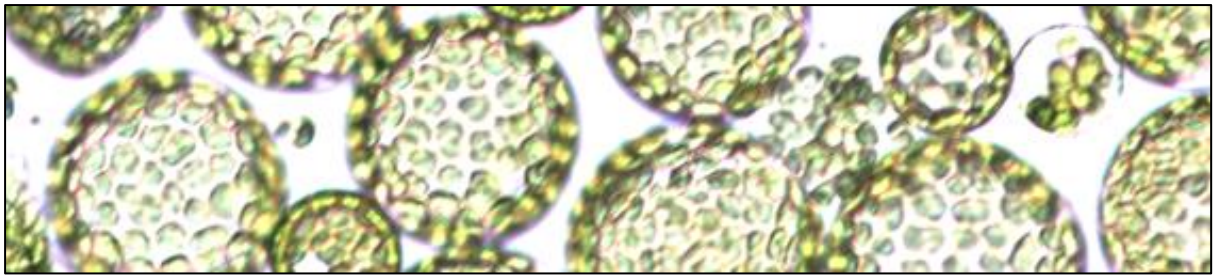
List of Abbreviations

%	percentage
ºC	degrees celsius
3AT	3-amino-1,2,4-triazole
5FOA	5-fluoroorotic acid
aa	amino acid
ADP	adenosine diphosphate
ADP-Glc	ADP-glucose
AGPase	ADP-glc pyrophosphorylase
Amp	ampicillin
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
bp	basepair
bya	billion years ago
CC	companion cells
cDNA	complementary DNA
CIN	cytoplasmic invertases
CO2	carbon dioxide
CWI	cell wall invertases
DNA	deoxyribonucleic acid
FPLC	fast protein liquid chromatography
Frc	fructose
Frc1,6BP	fructose 1,6-bisphosphate
Frc1,6BPase	fructose-1,6-bisphosphatase
Frc6P	fructose 6-phosphate
g	gram
g	gravity
GFP	green fluorescent protein
Glc	glucose
Glc1P	glucose 1-phosphate
IPTG	Isopropyl β- d-1-thiogalactopyranoside
GPP	UDP-glucose pyrophosphorylas
GST	glutathione S-transferase
GUS	glucuronidase
h	hour
H+	hydrogen
H2O	water
HA	hemagglutinin
HAD	haloacid dehalogenase
HRP	horseradish peroxidase
HXK	hexokinase
INV	invertase

kDa	kilo dalton
LC-MS	liquid chromatography mass spectrometry
M	molar
mg	milligram
min	minutes
ML	maximum likelihood
ml	milliliter
mya	million years ago
NCBI	national center for biotechnology information
ng	nanogram
O2	oxygen
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGM	phosphoglucomutase
Pi	inorganic phosphate
qPCR	real-time quantitative PCR
RNA	ribonucleic acid
RT	room temperature
SAM	shoot apical meristem
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE/CC	sieve element-companion cell complex
SNF	sucrose nonfermenting
SnRK1	SNF1-related protein kinase 1
SPP	sucrose phosphate phosphatase
SPS	sucrose phosphate synthase
SS	starch synthases
Suc6P	sucrose 6-phosphate
SUS	sucrose synthase
SUT	sucrose uptake transporter
TP	triose phosphates
TPP	trehalose 6_phosphate phosphatase
TPS	trehalose 6-phosphate synthase
TRE	trehalase
Tre6P	trehalose 6-phosphate
TreP	trehalose phosphorylase
TreS	trehalose synthase
TreT	trehalose glycosyltransferring synthase
TreY	maltooligosyltrehalose synthase
TreZ	maltooligosyltrehalose trehalohydrolase
UDP	uridine diphosphate
UDP-Glc	UDP-glucose
VIN	vacuolar invertases

w/v	weight per volume
WT	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ΔCt	threshold cycle number
µl	microliter
µm	micrometer (micron)



Chapter 1

General Introduction

Virtually all living creatures on this planet rely on photosynthesis, a process that liberates oxygen (O_2) while fixing atmospheric carbon dioxide (CO_2). The sugars formed during this process are central to primary carbon metabolism and have acquired essential regulatory functions over the course of evolution. Once taken up by cells, sugars can be stored, converted to structural polymers, partake in signaling cascades or enter central metabolism. Because plants are sessile, maintaining homeostasis requires extensive regulatory machinery. To this effect, the signaling function of sugars come into play, where they participate in regulating metabolism, growth and development (Rolland et al., 2006). This process involves sugar sensing, subsequent signal transduction and finally, target gene expression. Under conditions of sugar abundance, the expression of genes implicated in overall sink function (*e.g.* synthesis of starch, protein storage, plant growth) are stimulated. Under conditions of sugar depletion, gene expression towards photosynthesis and storage reserve breakdown are stimulated. Essentially, sugar signaling sends information to the nucleus regarding the overall energy status of the plant, allowing it to adapt accordingly.

One sugar central to primary carbon metabolism is sucrose, a disaccharide found exclusively in autotrophic organisms. Sucrose synthesis in plants take place in the cytosol in a tightly controlled manner, with the enzyme catalysing the first dedicated step, regulated post-translationally (Hardin et al., 2003). This control is necessary to balance out supply and demand within the plant. Sucrose degradation is mediated by one of two enzymes, invertases (INV) and sucrose synthases (SUS) and one part of this study focussed on the latter. SUS proteins are glycosyl transferases mostly active in sink tissues, where they cleave incoming sugar, setting the sink strength of the organ in question. The breakdown products can subsequently enter one of several pathways; for example, ones that generate energy or ones that synthesize complex

carbohydrates such as cellulose (Amor et al., 1995). Genetically modified plants with altered SUS activity show diverse phenotypes. For example, reduction of SUS activity in tomato mutants lead to plants with reduced complex carbohydrate synthesis, reduced growth and altered leaf morphology (Goren et al., 2017). Over-expressing *SUS* leads to an improved growth and an increase in the synthesis of complex carbohydrates, making them ideal candidates for the manipulation of crop plants (Nguyen et al., 2016). Sucrose synthases have been the subject of intensive research, however, certain aspects of their function and regulation remain unknown. For example, why have plants evolved two sucrose cleaving enzymes (SUS and INV) and how is sucrose degradation divided between these groups?

Another disaccharide of interest is trehalose, a sugar present in all kingdoms of life, thought to be more ancient in origin than sucrose. It accumulates to very high levels in some resurrection plants but is only found in trace amounts among the majority of land plants. Its precursor trehalose 6-phosphate (Tre6P) has been the subject of intense study the last few decades. Levels of this sugar phosphate were demonstrated to change in parallel to that of sucrose, with researchers proposing that it plays a role in communicating sucrose availability (Lunn et al., 2006). Mutants defective in specific steps of Tre6P metabolism present pleiotropic phenotypes ranging from defects in embryogenesis to seed set (Eastmond et al., 2002). Again, despite research efforts, certain aspects of Tre6P metabolism remain unknown. For example, where it is localized within cells and how it moves between compartments. The second part of this study focussed on the proteins involved in Tre6P synthesis, namely trehalose 6-phosphate synthases (TPSs). These proteins are divided into two classes, with class I proteins containing catalytically active proteins while class II polypeptides are inactive (Leyman et al., 2001; Lunn, 2007). Because of this, the majority of research has concentrated on class I enzymes, leaving class II isoforms under-researched. The focus of this study was on the class II TPS's.

Finally, the majority of research examining these proteins alongside sugar metabolism and signaling in general, have been conducted in vascular plants, leaving aspects thereof unresolved in non-vascular plants. Many years ago, the moss *Physcomitrella patens* was developed into a model plant for use in studies of evolutionary development and non-vascular plant biology. This bryophyte allows easy observation of mutations due to its dominant haploid life cycle, a feature that has attracted various studies into gene function in this plant. For these and other reasons, *P. patens* was chosen as the plant of choice to unravel aspects of SUS and TPS function in non-vascular plants.

Aim and Thesis Layout

The overall aim of this study was to characterize two gene families in *P. patens*, orthologs of which in vascular plants are implicated in sugar metabolism and signaling. We were interested in revealing whether gene function was conserved over the course of evolution, or whether it underwent neo-functionalization as a consequence of adapting to life on land. An overview of the two gene families and *Physcomitrella*, are given in Chapter 2 in the form of a literature review, together with an overview of the sugars sucrose and trehalose. The methodology used to characterize the two gene families are presented in Chapter 3, with the findings presented and discussed in Chapters 4 (*SUS* gene family) and 5 (class II *TPS* gene family). Finally, in Chapter 6, the findings from both experimental chapters are summarized and directives for future studies discussed.

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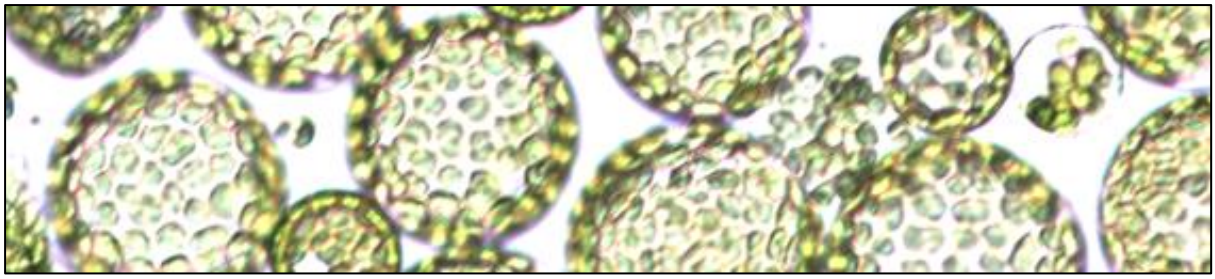

Chapter 2

Sucrose and Trehalose - A Literature Review

2.1. Introduction

Autotrophic plants have evolved the capability of converting light energy into reducing sugars to fuel their growth and development. Some of these sugars have been implicated in signaling processes, where they fulfil hormone-like functions in relaying information to the nucleus regarding resource availability, allowing the plant to adapt its metabolism to changing environmental circumstance. This, together with other mechanisms, allow these sessile organisms to respond optimally to biotic and abiotic stresses, or to make full use of advantageous conditions. Researchers are attempting to unravel the mechanisms that underlie these processes for two main reasons: firstly, to add knowledge to the field, and secondly to create plants superior in their ability to withstand stresses in order to feed the ever-growing population. The focus of this chapter will be on the sugars sucrose and trehalose. Although the roles of both of these have been extensively studied, aspects of their metabolism and signaling remain unresolved. Furthermore, most studies on these sugars were carried out in vascular plants, meaning that information on the roles they play in non-vascular plants is lacking. In this study, *P. patens* was used to examine sucrose and trehalose metabolism and signaling in a non-vascular plant and, therefore, the first section of this review is dedicated to this bryophyte.

2.2. A brief overview of plant evolution

Prior to the great oxygenation event approximately 2.3 billion years ago (*bya*), the Earths' atmosphere was anaerobic. At that point, oceanic cyanobacteria started producing oxygen *via* oxygenic photosynthesis, where a water molecule is used as an electron donor, being split to release O_2, protons and electrons (Blankenship and Hartman, 1998). As a result, atmospheric carbon dioxide became fixed, while O_2 was simultaneously released into the atmosphere (Blankenship, 2010, 2001; Nelson, 2011). Initially the free O_2 was captured by dissolved iron and organic matter, however, over time these sinks became saturated, allowing atmospheric O_2 levels to rise. It took about 1 billion years for this increase to allow eukaryotic life to develop (Hohmann-Marriott and Blankenship, 2011). During the course of evolution, eukaryotes acquired plastids and mitochondria from photosynthetic cyanobacteria and free living *α*-proteobacteria respectively, *via* endosymbiosis (Margulis, 1981). This led to an increase in both cell number and cellular complexity of organisms (Reyes-Prieto et al., 2007). The earliest plants evolved in the ocean where they were restricted to the upper layers where light could penetrate. According to the earliest fossil evidence, plants colonised the land about 470 million years ago (*mya*), evolving from green algae that migrated to moist terrestrial areas (Bateman et al., 1998; Fiz-Palacios et al., 2011; Qiu and Palmer, 1999; Waters, 2003). In order to move to a terrestrial environment, early land plants evolved features that allowed them to withstand desiccation. These early adaptations included the procurement of stomata, a waxy cuticle and gametangia. Furthermore, in order to increase nutrient acquisition from the soil, these early land plants formed symbiotic associations with fungi (Bidartondo et al., 2011; B. Wang et al., 2010). Approximately 420 *mya*, plants evolved the means to translocate water and organic matter throughout their body, through the formation of vasculature (Ligrone et al., 2012; Lucas et al., 2013). This allowed them firstly to grow upright by providing structural support and, secondly to move away from moist environments, by decreasing their dependence on proximate water to remain hydrated. Circa 375 *mya*, these vascular plants evolved structures (seeds) that protect and nourish the developing embryo (Davis and Schaefer, 2011; Lucas et al., 2013). As a consequence, these early seed plants (gymnosperms) were able to colonize even dryer regions, as their embryos were protected from desiccation. Finally, approximately 140 – 180 *mya*, angiosperms appeared. These plants have reproductive features referred to as flowers (Soltis et al., 2008), and within these seeds are carried within ovaries. These develop into fruit allowing for seed dispersal. Originally, these seeds had two cotyledons (dicot species) which later fused and lead to the development of monocot species.

2.3. The model plant *Physcomitrella patens*

Mosses serve as ideal models for a variety of research applications, with *Physcomitrella patens* developed as the model of choice (Knight et al., 2009). Their developmental pattern is simple, they are small in size, easily cultured, possess many features similar to those of vascular plants, respond to environmental stimuli and hormonal cues in a similar fashion and their dominant life cycle consists of the haploid gametophyte stage (Cove et al., 1997; Schaefer and Zrÿd, 2001). The most widely used Gransden ecotype was collected in the UK in 1962 (Engel, 1968). Prior to the development of efficient genome editing techniques, *Physcomitrella* was one of the easiest plants to manufacture knockout mutants, due to the high frequency of homologous recombination that occurs in its genome. This made the isolation of mutants easy due to the haploid nature of its dominant gametophyte stage, allowing recessive traits to be observed directly (Knight et al., 2009). Since the development of protocols for *Physcomitrella* transformation, and the discovery of its native homologous recombination system, it has become the only multicellular photosynthetic eukaryote with efficiencies for gene targeting similar to those observed for *Saccharomyces cerevisiae* (Schaefer and Zrÿd, 2001). A similar system has been developed for the liverwort *Marchantia polymorpha*, however, homologous recombination was only observed in 2% of stable transformants (Ishizaki et al., 2013). Furthermore, the availability of its nuclear, chloroplast and mitochondrial genomes allow for comparative genomic studies examining land plant evolution (Rensing et al., 2008).

2.3.1. The haploid-dominant life cycle of *P. patens*

P. patens growth and development consists of a dominant haploid autotrophic gametophyte stage and a minor diploid heterotrophic sporophyte stage (Figure 2.1). The gametophyte consists of two developmental stages, the production of protonema followed by the development of gametophores. Protonema consists of a filamentous network of chloronemal and caulonemal filaments which differ in how densely their cells are packed with chloroplasts and how the individual cells are separated (Cove et al., 1997; Schaefer and Zrÿd, 2001). From the caulonemal cells, a photosynthetic non-vascular stem-like structure called a bud develops, which later differentiates into the gametophore. The gametophore carries leaf-like structures along its stem and, at its base rhizoids which function as root-like structures to anchor the gametophore in place. In addition, the gametophore carries gametangia (sexual organs), whose development is induced by a short daylight regime and a drop in temperature. Both male and female gametes are produced by the same plant, making moss monoecious. Male gametes,

produced by the antheridia, require water to swim into the archegonia where the female gametes reside for fertilization. Once fertilized, the zygote develops into a diploid sporophyte that carries a spore capsule where meiosis takes place to produce approximately 4000 haploid spores. Finally, released spores will germinate to produce further gametophytes, allowing the cycle to continue.

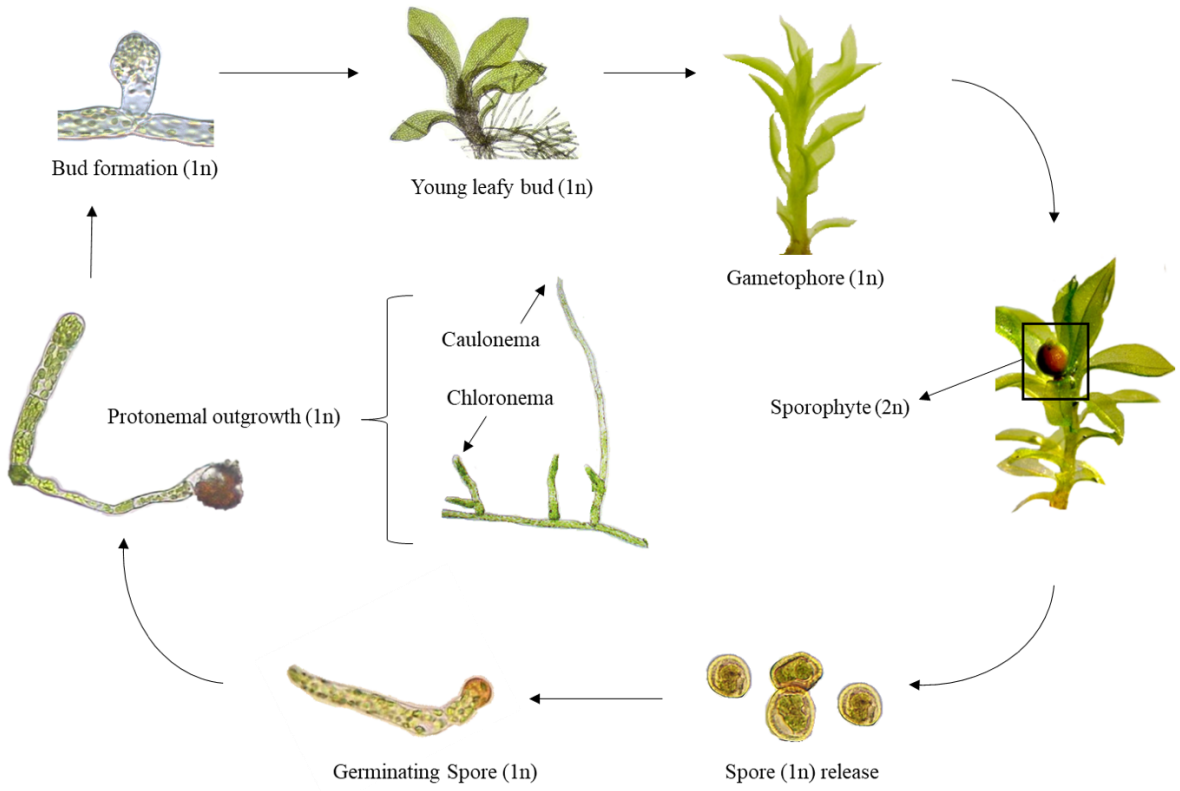

Figure 2.1. The life cycle of *Physcomitrella patens*. Haploid spores released from diploid sporophytes germinate and start differentiating into protonemal tissue, made up of chloronemal and caulonemal cells. From the protonemal tissue, buds develop that differentiate into leafy buds and finally gametophores. Within the gametophores, archegonia and antheridia (not shown) develop in the apex, where fertilization ultimately takes place to produce the diploid sporophyte. The sporophyte eventually releases spores and the cycle continues. *Illustration made using a compilation of images obtained from open source articles, which include: Menand et al., 2007; Prigge and Bezanilla, 2010; Roberts et al., 2012.*

2.3.2. Evolution and phylogenetic position

Land plant divergence started after colonisation 450 *mya*, with the first separations differentiating the bryophytes (mosses, liverworts, hornworts) from the remaining land plants (de Lucas et al., 2008; Rensing et al., 2008). The mosses in particular occupy an ideal evolutionary position to help in understanding processes that differentiate algae from angiosperms as they last shared a common ancestor with both at the time of land colonisation (Lang et al., 2018; Reski et al., 2015; Schaefer and Zrÿd, 2001). By comparing the genome sequence of *P. patens* to angiosperms and aquatic single celled algae, it is possible to reconstruct how the genetic makeup of plants evolved during the colonization of land and, subsequently, how biological processes evolved as a consequence of this change (de Lucas et al., 2008; Schaefer and Zrÿd, 2001). Comparing the differences between bryophyte and angiosperm development can further help to infer alterations during early stages of land plant evolution (Rensing et al., 2008). Bryophytes are characterized by a dominant haploid gametophytic generation, while modern angiosperms possess a dominant diploid sporophyte generation (Schaefer and Zrÿd, 2001). The move to a principal diploid generation was a puzzling one. The "masking hypothesis" proposes that diploidy offers redundancy, protecting the individual against the immediate effects of harmful mutations (Kondrashov and Crow, 1991; Perrot et al., 1991). However, it is not without consequence, as it masks the accumulation of beneficial recessive alleles over time, decreasing the fitness of populations over the long term (Schaefer and Zrÿd, 2001).

2.3.3. Photosynthate transport in *P. patens*

As mentioned above, *P. patens* is a non-vascular plant, meaning that photosynthate transport throughout the plant body does not utilize phloem elements. However, mosses do share some anatomical features with vascular plants. The gametophytic stem contains a central water conducting channel, made up of xylem analogues called hydroids (Sakakibara et al., 2003). Furthermore, it also contains nutrient conducting cells analogous to phloem sieve cells, called leptoids (Behnke and Sjolund, 2012; Ligrone et al., 2000). The cell walls of these leptoids contain plasmodesmata scattered throughout, hinting at a symplasmic mode of photosynthate transport in the gametophyte (Regmi et al., 2017). In this fashion, molecules pass through the plasmodesmata between cells, forming a conduit for photosynthate transport (Raven, 2003; Regmi et al., 2017; Reinhart and Thomas, 1981).

2.3.4. The genome of *P. patens*

Plants store genetic information in three compartments, mitochondria, plastids and the nucleus. All three of these genomes have been sequenced in *Physcomitrella*, providing a wealth of information related to plant evolution. Although comparative genomics mostly focusses on the nuclear genome, examination of the other two has provided insight into genes that have migrated from the chloroplast to the nucleus and how the genome structure of mitochondria evolved. The nuclear genome was sequenced *via* whole-genome shotgun sequencing in 2005, with the draft sequence published in 2008 (Rensing et al., 2008). Comparative genomics involving this has highlighted the major changes plants underwent when they colonized land. Genome annotation is still progressing and sequence data can be accessed at https://phytozome.jgi.doe.gov (Reski et al., 2015; Zimmer et al., 2013). It consists of approximately 500 mega base (Mb) with the genetic information spread across 27 chromosomes (Reski et al., 1998). Approximately 36 000 protein-coding genes were predicted for *v1.1* of the genome. A follow up assembly in *v3.1* predicted 35 307 protein coding genes, with only 78% being functionally annotated (Lang et al., 2018). Furthermore, gene ontology analyses revealed an over-representation of genes involved in metabolism. Indeed, in v1.2, approximately 70 – 80% of genes were predicted to be involved in metabolism, considerably more than the 10 – 44% observed for *Arabidopsis*. This suggested either the existence of metabolic pathways absent in flowering plants, or duplication events of genes related to carbon metabolism (Thelander et al., 2009). It has in fact been determined that *P. patens* is a "paleopolyploid", with the genome duplication event in question, dating back between 30 and 60 *mya* (Rensing et al., 2008). In that study they were able to demonstrate that unlike *Arabidopsis*, which retained genes involved in transcriptional regulation (Seoighe and Gehring, 2004), *P. patens* retained mostly metabolism related genes (Rensing et al., 2008). In addition to the availability of its sequenced genome, a platform (https://genevestigator.com) has been developed where researchers can access published transcriptome data sets, allowing the analyses of transcript abundance under specific conditions, treatments or developmental stages (Hiss et al., 2014; Hruz et al., 2008; Reski et al., 2015). Taken together, the *Physcomitrella* genome and its related transcriptome, serve as rich resources for comparative and functional genomics.

2.3.5. The ease of gene targeting in *P. patens*

To understand how genes, and the proteins they encode, function in intact organisms researchers need to introduce precise alterations (*e.g.* knock-out, over expression or knock-in) within specific components of gene networks (Cove et al., 1997; Schaefer and Zrÿd, 2001). In diploid organisms, this can prove laborious, due to the need for back crosses to obtain homozygous mutants for recessive traits to be observed. Thanks to its native homologous recombination system, *Physcomitrella* allows targeted transgenesis to take place at the original chromosomal location. In addition to coding sequences, mutagenesis can be applied to promoters, regulating elements and non-coding sequences. Furthermore, due to its dominant haploid gametophyte stage, changes brought on by mutagenesis can be observed immediately (Reski et al., 2015; Schaefer and Zrÿd, 2001). Spatial and temporal gene expression and protein localization can also be monitored *in vivo* by transforming *Physcomitrella* with vectors that carry native sequences fused with cytological markers (GUS or GFP) for observation of gene expression or subcellular protein localisation. Over the years, optimized protocols have been developed for the isolation, transformation and regeneration of moss protoplasts, with PEG-mediated transformation the method of choice (Reski et al., 2015). Taken together, *Physcomitrella* has several features that make it an ideal model plant to work with.

2.4. Non-reducing disaccharides

Non-reducing disaccharides cannot donate electrons, rendering them unable to act as reducing agents. This is due to their constituent monosaccharides being linked at their reducing ends, producing stable, energy rich disaccharides. Because they are stable molecules, they tend to be used as a soluble energy source, able to be translocated by various organisms (with the exception of vertebrates). In addition, they can also function as protective compatible solutes when plants experience abiotic stress. Among these sugars, sucrose and trehalose have played a central role in the evolution of life, thanks to their chemical properties. In plants sucrose is often the main translocated sugar and is, therefore, present in large amounts. Trehalose on the other hand is found only in trace amounts, with the exception of some resurrection plant species. In the following sections, sucrose and trehalose metabolism (and signaling) will be discussed in more detail, with a focus on the enzymes that participate in their metabolic pathways.

2.5. Sucrose

Sucrose is a carbohydrate that is translocated from the source to sink tissues in most plants (Lunn, 2008; Ruan, 2012). Figure 2.2 illustrates the pathways for sucrose synthesis, transport and catabolism in vascular plants, which will be discussed briefly below. During the day, CO_2 is fixed within the chloroplasts of source leaves, yielding triose-phosphates (TPs) as end products. The TPs are subsequently exported to the cytosol in exchange for inorganic phosphate (Pi) *via* a triose-phosphate:phosphate translocator (Lunn, 2008). In the cytosol, aldolase converts TPs to fructose 1,6-bisphosphate (Frc1,6BP). The latter gets dephosphorylated to fructose 6-phosphate (Frc6P) through fructose-1,6-bisphosphatase (Frc1,6Bpase) (Ruan, 2014). From here, phosphoglucomutase (PGM) and phosphoglucose isomerase (PGI) equilibrate Frc6P, glucose 1-phosphate (Glc1P) and glucose 6-phosphate (Glc6P) for subsequent use in UDP-glucose (UDP-Glc) synthesis by UDP-glucose pyrophosphorylase (GPP). These hexose-phosphates are used for the synthesis of sucrose by sucrose phosphate synthase (SPS) and sucrose phosphate phosphatase (SPP). Finally, these reactions produce Pi, which can be returned to the chloroplast in exchange for TPs for continued sucrose synthesis.

Sucrose can be loaded into the phloem apoplasmically (*via* sugar transporters) or symplasmically (*via* plasmodesmata) through the sieve element-companion cell complex (SE/CC) (Ruan, 2014). Once it arrives at sink tissues, sucrose needs to be unloaded. This loading and unloading generates turgor pressure which drives the mass flow of photo-assimilates, nutrients, water and signaling molecules between source and sink tissues. As in loading, sucrose can be unloaded apoplasmically or symplasmically. Apoplastic unloading involves cell wall invertases (CWI) that hydrolyse sucrose into glucose (Glc) and fructose (Frc) prior to being taken up into the cytosol. Sucrose unloaded symplasmically via plasmodesmata or taken up by SUTs can be degraded by either cytoplasmic invertases (CIN) or sucrose synthases (SUS). Furthermore, sucrose can be transported into vacuoles where it can be stored or degraded by vacuolar invertases (VIN). The hexoses generated by these degradative enzymes can be used in glycolysis to fuel cellular processes and/or as the building blocks for the synthesis of polymers like starch, cellulose, callose and proteins.

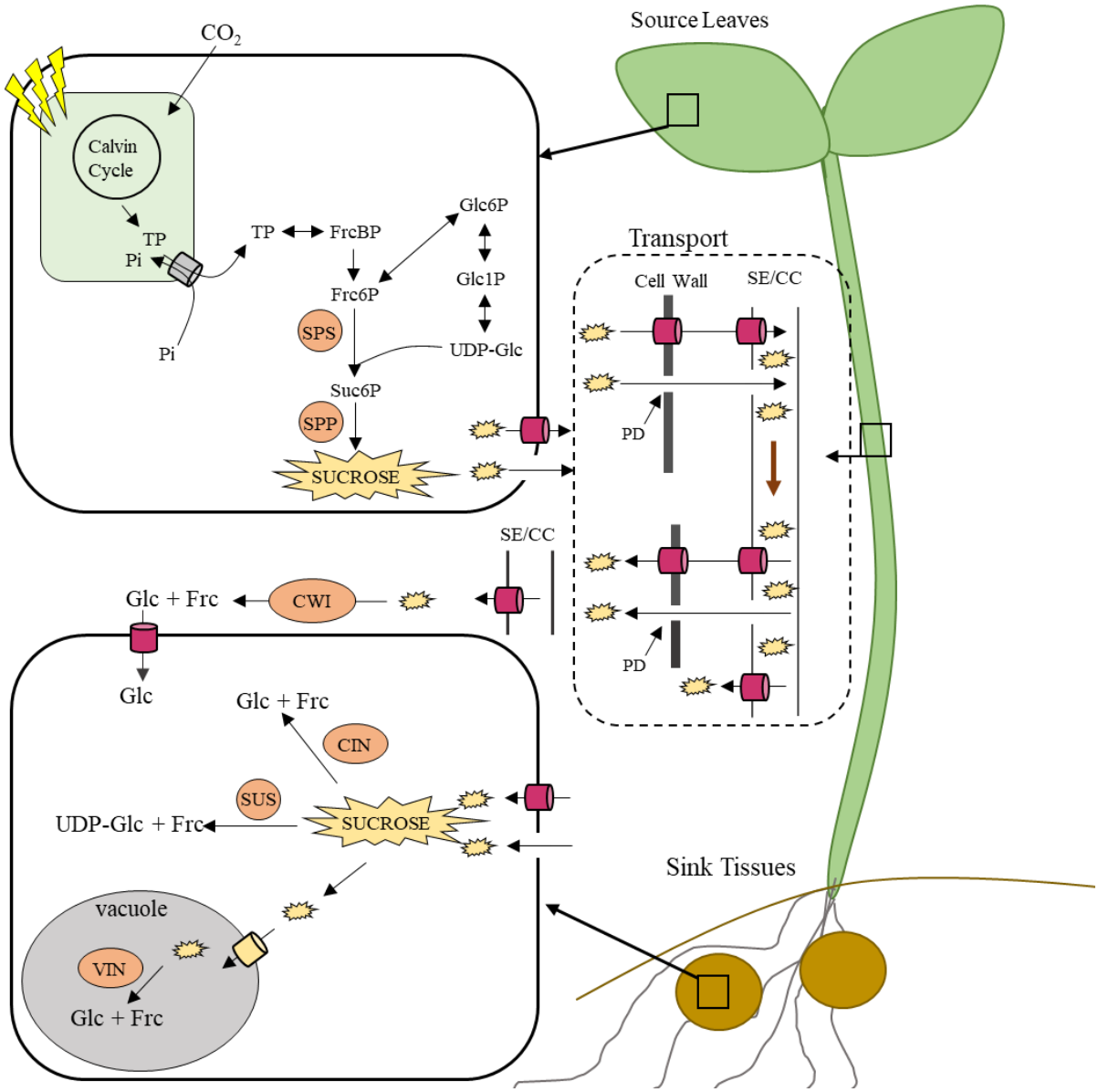

Figure 2.2. An overview of sucrose synthesis, transport and breakdown in photosynthetic plants. Using light, CO_2 is fixed to produce TPs (*via* the Calvin cycle) within chloroplasts. These TPs are exported to the cytosol where they are used in the synthesis of sucrose *via* the SPS/SPP pathway. Sucrose can then be loaded into the phloem for transport to sink tissues, where it is unloaded. Within the cytosol, sucrose can be cleaved by SUS and/or CIN or transported to the vacuole for degradation by VIN. Abbreviations: SE/CC – sieve element/companion cell; TP – triose phosphates; FrcBP – fructose bisphosphate; Frc6P – fructose 6-phosphate; UDP-Glc – UDP-glucose; Glc1P – glucose 1-phosphate; Glc6P – glucose 6-phosphate; Glc – glucose; Frc – fructose; Suc6P – sucrose 6-phosphate; CWI – cell wall invertase; CIN – cytosolic invertase; VIN – vacuolar invertase; SUS – sucrose synthase; PD – plasmodesmata; Pi – inorganic phosphate (*Image adapted from Ruan, 2014*).

2.6.1. Sucrose is synthesized by the combined actions of two enzymes

As shown in Figure 2.2, sucrose is synthesized within the cytosol *via* SPS and SPP. Here, SPS synthesizes sucrose 6-phosphate (Suc6P) from UDP-Glc and Frc6P, which is then dephosphorylated to sucrose by SPP. SPS is an important regulator of sucrose synthesis, being activated under conditions of osmotic stress and deactivated by light *via* protein phosphorylation (Huber and Huber, 1996). In *Arabidopsis* leaves, *AtSPS* is co-expressed with genes involved in sucrose export from mesophyll cells to the apoplasm, namely *AtSWEET11* and *12* for phloem loading (Chen et al., 2012). Sucrose synthesis is not restricted to source tissues as there is a constant cycle of sucrose synthesis and degradation in heterotrophic tissues. This is thought to add flexibility within the tissue which may need increased sucrose for storage or intercellular transport.

2.5.2. Sucrose degradation *via* a plethora of invertases

Upon phloem unloading in sink tissues, sucrose can be cleaved into different products depending on the enzyme involved. Invertases catalyse the irreversible hydrolysis of sucrose into Glc and Frc. Invertases are grouped according to their pH optima and subcellular localization (Sturm, 1999). These groups include CWI, VIN and CIN. Cell wall invertases are key regulators of carbon partitioning and are generally expressed in sink tissues where phloem unloading takes place. Maize *cwi2* ethyl methane sulfonate (EMS) mutants with altered enzyme activity demonstrate the importance of this enzyme in sink development, with these lines having a miniature seed phenotype (Cheng et al., 1996). Similarly, rice knock-out or overexpression lines of *CWI2* show respectively reduced or improved grain yield (Wang et al., 2008). Similarly, silencing *CWI1* expression in tomato resulted in an inhibition of seed and fruit development, while overexpression resulted in the opposite (Zanor et al., 2009).

Vacuolar invertases are active in hexose accumulating tissues. For example, *VIN* transcripts are found in tomato fruit that accumulate hexoses but not in fruit that accumulate sucrose (Klann et al., 1993). Silencing *VIN1* expression in tomato leads to fruit that accumulate sucrose instead of hexoses (Klann et al., 1993). By cleaving sucrose into Glc and Frc, these invertases effectively double the osmotic potential. This observation, alongside a demonstration of high VIN activity in rapidly expanding tissues has implicated these enzymes in cell expansion (L. Wang et al., 2010). One tissue type that grows *via* cell expansion is that of roots (Dolan and Davies, 2004). It has been demonstrated that *Arabidopsis vin2* mutants have a short root

phenotype (Sergeeva et al., 2006), while complementation of this mutant with a homolog from cotton, *GhVIN1*, reversed the phenotype (L. Wang et al., 2010).

Cytosolic invertases can be divided into a number of subgroups. The α-group localize to intracellular organelles, whereas the β-group localize to the cytoplasm and sometimes the nucleus (Murayama and Handa, 2007). The precise nature of their function is not yet resolved; however, it has been proposed that they play a role in root and reproductive development. An *Arabidopsis cin7/cin9* double knock-down line showed reduced root growth ascribed to abnormal cell expansion (Barratt et al., 2009). Similarly, a *Lotus japonicus ljinv1* mutant with a premature stop codon and missense mutations introduced, had a negative effect on root growth, pollen development and flowering (Welham et al., 2009). Homozygous lines showed a stark reduction in shoot and root growth, a lack of pollen in stamens and altered cellular development.

2.5.3. The duality of sucrose synthases

Interestingly, plants encode a second class of enzymes that partake in sucrose degradation, the sucrose synthases (SUS). However, unlike INV, SUS catalyses both the degradation and synthesis of sucrose. The direction of the reaction depends on the pH of the surrounding environment, with a low pH range favouring the degradative reaction and a high pH the synthetic reaction. In the synthesis reaction, SUS utilizes Frc and UDP-Glc as substrates. In the degradation reaction SUS can utilise either UDP or ADP (albeit with lower affinity than UDP) as substrates alongside sucrose to release either UDP-Glc or ADP-Glc. Sucrose synthase activity is widely regarded as a marker for sink strength (Chey and Nelson, 1976; Counce and Gravois, 2006; Craig et al., 1999; Zrenner et al., 1995), which will be elaborated on in this section, together with other aspects thereof in plants.

2.5.3.1. SUS gene families and characteristics of the protein

Sucrose synthase genes are widespread in plants and are differentiated into three clades, namely *SUSI*, *SUSII* and *SUSIII*. Different species contain different numbers of these genes. Five *SUS* genes are encoded in grape (Zhu et al., 2017), six in *Arabidopsis*, rice and tomato (Baud et al., 2004; Goren et al., 2017; Hirose et al., 2008) and seven in cotton (Chen et al., 2012). Other plants contain significantly more, with tobacco encoding fourteen (Wang et al., 2015) and poplar fifteen (An et al., 2014). SUS proteins are glycosyltransferases belonging to the glycosyltransferase-4 subfamily. Their monomers can form homotetramers (Schmölzer et al.,

2016) and heterotetramers (Duncan et al., 2006; Guerin and Carbonero, 1997), depending on the plant species. Sucrose synthases have two domains, each with a distinct function. The N-terminal domain is implicated in cellular targeting while the C-terminal domain possesses glycosyltransferase activity (Zhang et al., 2011). Furthermore, SUS proteins have several sites for protein phosphorylation and protein degradation (Hardin et al., 2003). Phosphorylation of certain residues leads to protein association to the membrane. Sucrose synthase protein localization is not restricted to the cytosol, even though it was first identified in these fractions (Macdonald and ap Rees, 1983; Nishimura and Beevers, 1979). The enzymes are normally considered soluble in the cytosol due to the globular nature of the protein. Evidence for plasma membrane association has come from cotton fibres, where roughly 50% of SUS is localized there (Amor et al., 1995). Furthermore, it was found that more than half of the total SUS activity in cotton fibres (*Gossypium hirsutum*) is associated with the plasma membrane, possibly channelling carbon directly to cellulose and/or callose synthases on site (Amor et al., 1995). Tobacco pollen tube SUS proteins localize to both the cytosol and plasma membrane (Persia et al., 2008). Association to the plasma membrane has been proposed to depend on N-terminal phosphorylation. For example, in tobacco, phosphorylation decreases membrane association, while dephosphorylation promotes it (Winter et al., 1997). It has also been demonstrated that reversible phosphorylation of both the membrane and cytosolic forms, promotes interaction with the actin cytoskeleton (Winter et al., 1998). This degree of post-translational regulation hints at the importance of SUS in plant metabolism.

Moreover, SUS has also been shown to localize to cell walls in the case of cotton (Salnikov et al., 2003), vacuolar membranes in red beet (Etxeberria and Gonzalez, 2003), mitochondria of poplar (Konishi et al., 2004) and plastids of *Arabidopsis* seeds (Núñez et al., 2008). The detection of SUS protein in the membrane fraction of protein extracts, enriched in cytoskeletal polymers, suggested that SUS may interact with components found in the cytoskeleton (Winter et al., 1998). Co-immunoprecipitation experiments demonstrated that cytosolic SUS interacts with G-actin (Winter et al., 1998). These diverse locations imply an important role in many cellular processes.

2.5.3.2. The role of SUS in establishing and/or maintaining sink strength

The ability of a given organ or tissue to import reduced carbon (*e.g.* sucrose) is referred to as 'sink strength', and relies on the size and activity of the organ (Ho, 1988). Phloem unloading and subsequent sucrose degradation contribute to sink strength. Using promoter-GUS fusions, SUS expression has been detected in the phloem of several plant species. *Arabidopsis SUS5* and *SUS6* are phloem specific (Barratt et al., 2009). However, SUS activity is not restricted to phloem unloading zones, as it has been localized to phloem loading zones (*e.g.* leaves) (Nolte and Koch, 1993; Regmi et al., 2016) as well, suggesting a more general role for these proteins. A study using immunohistochemistry to localize SUS activity in maize and citrus found the enzyme to be specifically associated to phloem companion cells (CC) of both source and sink tissues (Nolte and Koch, 1993). This suggests involvement in both the loading and unloading of sucrose, possibly maintaining the gradient necessary for assimilate translocation between source and sink tissues. Within the phloem, SUS could be involved in maintaining an equilibrium between sucrose and its breakdown products, which are utilized for energy production in CC and as substrates for complex carbohydrate synthesis (Nolte and Koch, 1993). Sucrose loading in the phloem occurs via a sucrose-H^+ co-transporter and takes place against a concentration gradient energized by the plasma membrane H^+-ATPase (Giaquinta, 1977; Riesmeier et al., 1993; Slone and Buckhout, 1991). The H^+-ATPase relies on steady supply of ATP, which could possibly be provided by the degradation of a portion of sucrose within the phloem of CCs by SUS. To this effect, a correlation has been found between SUS activity and an increase in carbon flux through the respiratory pathway (Black et al., 1987; Farrar and Williams, 1991).

Evidence suggests that SUS is the dominant enzyme in sink tissues responsible for sucrose degradation (Morrell and Rees, 1986; Wang et al., 1994). Using heat shock treatment to distinguish SUS activity from acid INV activity, it was demonstrated that SUS was the main enzyme employed by tomato fruit to cleave imported sucrose, which establishes and maintains the sink strength of the organ (Wang et al., 1993). In legumes, SUS activity was found to increase rapidly during nodule development, with this increase most likely due to phloem unloading during nitrogen fixation that would supply the rhizobial bacteria with carbon skeletons from sucrose breakdown, in the form of malate (Thummler and Verma, 1987). The role of SUS in nitrogen assimilation was demonstrated in pea *rug4* mutant plants, whose nodules have an 85% reduction in SUS activity (Craig et al., 1999). Even though *rug4* nodules contain metabolically active rhizoids, their nitrogen content was low compared to WT nodules,

demonstrating the involvement of SUS in nitrogen assimilation. Maize *sh* knock-out lines display a 90% decrease in endosperm SUS activity which resulted in a reduction in starch content and a subsequent shrunken or collapsed kernel phenotype (Chey, 1981; Chey and Nelson, 1976). A similar phenotype was observed for the pea *rug4* mutant, which has reduced SUS activity in the developing embryo. These plants presented with a wrinkled-seed phenotype coupled to reduced starch content (Craig et al., 1999). Furthermore, transgenic potato lines with suppressed SUS activity in their tubers presented with a strong inhibition of starch accumulation, strengthening the hypothesis that SUS is a major determinant of tuber sink strength (Zrenner et al., 1995). The *Arabidopsis sus2* and *sus3* single mutants showed an alteration in metabolism coupled to a decrease in transitory starch accumulation in developing seeds; interestingly however, seed phenotype was unaffected (Angeles-Núñez and Tiessen, 2010). Furthermore, no visible seed-related phenotypes were observed in either double (*sus2, sus3*) or quadruple mutants (*sus1, sus2, sus3, sus4*) (Barratt et al., 2009; Bieniawska et al., 2007), suggesting that in *Arabidopsis* at least, SUS is unimportant for sink strength in seeds.

2.5.3.3. SUS has been linked to starch synthesis

Pathways for starch synthesis differ between autotrophic and heterotrophic tissues. In autotrophic tissues, starch is synthesized within plastids from a pool of TPs converted into Frc1,6-BP, which is subsequently dephosphorylated to Frc6P for conversion to Glc6P. Glc1P is derived from the latter which can ultimately be used for the synthesis of ADP-Glc *via* ADP-Glc pyrophosphorylase (AGPase), the substrate for starch synthesis *via* starch synthases (SS). This pathway does not require sucrose breakdown and hence, SUS is not expected to be important for leaf starch synthesis. As mentioned above however, SUS can utilise ADP instead of UDP, yielding Frc and ADP-Glc (Baroja-Fernández et al., 2003). This has led to the proposition that SUS isoforms may synthesise ADP-Glc in the cytosol (Baroja-Fernández et al., 2009, 2004; Muñoz et al., 2005), which is then imported into the chloroplast to fuel starch synthesis by an unknown transporter (Bahaji et al., 2014). Evidence to support this comes from analysis of ADP-Glc levels in the leaves of *Arabidopsis* mutants unable to synthesis starch through loss of enzymes essential for plastidial ADP-Glc biosynthesis (Bahaji et al., 2011; Caspar et al., 1985; Lin et al., 1988; Muñoz et al., 2005). ADP-Glc amounts were found to be similar in these mutants to the WT, suggesting that another enzyme acts as a source of ADP-Glc in leaves (Bahaji et al., 2014; Muñoz et al., 2005). Furthermore, when *SUS* was overexpressed in tobacco, an increase in leaf starch content was observed (Nguyen et al., 2016).

However, this theory does not explain why reductions in plastidial ADP-Glc synthesis essentially eliminates starch synthesis (Caspar et al., 1985; Lin et al., 1988; Yu et al., 2000).

With regards to heterotrophic tissues, much evidence that links SUS to sucrose degradation fuelling starch accumulation. *SUS* overexpression in potato tubers leads to an increase in ADP-Glc, UDP-Glc and starch levels (Baroja-Fernández et al., 2009). Furthermore, mutants or transgenic plants with reduced SUS activity in maize endosperm, carrot taproots, pea seeds, potato tubers and *Arabidopsis* seeds accumulate decreased amounts of starch (Angeles-Núñez and Tiessen, 2010; Chey and Nelson, 1976; Craig et al., 1999; Tang and Sturm, 1999; Zrenner et al., 1995).

2.5.3.4. SUS and the role it plays in oxygen deficient environments

Low-oxygen stress can lead to a reduction in plant growth and results from either environmental (*e.g.* flooding) or developmental (*e.g.* rapidly growing tissues that consume a lot of oxygen) circumstances. Under these conditions, ATP production is halted due to a block in the mitochondrial electron transport chain, where oxygen serves as the terminal acceptor. Under these conditions plants adapt by regulating the expression of metabolic enzymes together with regulating their activities (Fukao and Bailey-Serres, 2004). One such enzyme is SUS. Transcript abundance for *SUS* has been shown to increase due to oxygen deficiency across a variety of plant species, for example *Arabidopsis* (Baud et al., 2004), maize (McCarty et al., 1986) and potato (Biemelt et al., 1999). Potato tubers of *SUS* antisense lines are more sensitive to low-oxygen conditions compared to control lines (Biemelt et al., 1999) while the *Arabidopsis sus1sus4* double mutant is more sensitive to flooding compared to controls (Bieniawska et al., 2007). These studies suggest that certain SUS proteins are important for metabolism in oxygen-deficient conditions. To this effect, sucrose breakdown *via* SUS is a more energy efficient reaction compared that catalysed by INV, since it saves two ATP molecules per sucrose molecule catabolized (Guglielminetti et al., 1995).

2.5.3.5. SUS function in the shoot apical meristem

SUS is also proposed to be involved in shoot apical meristem (SAM) development, a sink that acquires sucrose from the phloem. Promoter-GUS fusions revealed *SUS4* expression in the SAM of tomato lines (Goren et al., 2017). Half of the *SUS* genes in tomato, including *SUS4*, are expressed in meristems and primordia throughout development (Pien et al., 2001). Furthermore, *SUS4* transcript abundance around the SAM increases following sucrose and Glc treatment. Suppression of three *SUS* genes in tomato, resulted in plants with altered cotyledon and leaf morphology together with a change in the expression of auxin-related genes in the SAM (Goren et al., 2017), suggesting some regulatory function for SUS and sucrose.

2.6. Trehalose

Trehalose is a widely occurring sugar present in life forms ranging from bacteria to insects and plants. Until the mid 1990's it was thought to be absent in plants (with the exception of desiccation tolerant resurrection species), however, it was later shown that this was due to its accumulation at very low concentrations and the limits of detection of the technology used. As detection methods improved, it became possible to accurately detect trehalose and it was described to be present in a variety of plant species (Eastmond et al., 2002; Roessner et al., 2000; Roessner-Tunali et al., 2003; Vogel et al., 2001). Even after its initial detection in plants, researchers still ascribed little importance to it as it was found to be present in only micromolar concentrations (Müller et al., 2001). However, the discovery of genes encoding active proteins involved in trehalose synthesis in *Arabidopsis* (Blázquez et al., 1998; Vogel et al., 1998) allowed examination of its function. Expression of microbial genes of trehalose metabolism in plants revealed marked phenotypic changes compared to wild-type (WT), showing that interfering with trehalose metabolism can have far-reaching effects (Goddijn et al., 1997; Pilon-Smits et al., 1998; Romero et al., 1997). This indicated that it plays a role in sugar signaling as it does in microorganisms, such as yeast.

2.6.1. The molecule, its properties and applications in industry

Trehalose consists of two glucose moieties linked by a α-α-1, 1-glycosidic bond. Due to its unreactive nature, it is very resistant to heat, pH and chemical reactions. Moreover, its glycosidic link has a very low bond energy (Paiva and Panek, 1996) and it is only under extreme hydrolytic conditions, or in the presence of the enzyme trehalase, that trehalose dissociates into its constituent monosaccharides. These properties make it a useful stabilizer and hence an

attractive sugar utilized in industry. Much research has shown that trehalose acts as a powerful protectant of membranes, proteins, cells and organs for transplants (Paiva and Panek, 1996). In the pharmaceutical industry, it has been shown to reduce protein aggregation in Huntington disease mouse models (Tanaka et al., 2004), increase the survival rates of mammalian cells during cryopreservation (Eroglu et al., 2000), and improve cognitive abilities in Alzheimer's disease mouse models (Portbury et al., 2017). In cosmetic products, it serves mainly to protect skin and hair from dehydration under dry conditions. In the food industry, trehalose is added to food products prior to drying, which protects it from denaturation and a subsequent loss in flavour (Colaco et al., 1994). Furthermore, labile enzymes used in molecular biology (*e.g.* T7 DNA polymerase, T4 DNA ligase and restriction endonucleases) have been shown to withstand prolonged exposure to high temperatures when dried in the presence of trehalose, with no loss in activity (Paiva and Panek, 1996).

2.6.2. Trehalose is involved in widespread biological processes

Trehalose function varies from organism to organism. It has been shown to serve as an osmolyte that aids in desiccation, osmo-, thermo- and ethanol tolerance, to act as a storage carbohydrate in blood and to control the flux of glucose into glycolysis to name but a few (Crowe et al., 1984; Takayama and Armstrong, 1976). In micro-organisms trehalose can serve as a metabolic intermediate, used in glycolipid synthesis or serve as a structural molecule (Elbein, 1974). In some insects, trehalose serves as an energy source needed for flight (Wyatt and Kale, 1957) while other organisms accumulate trehalose in response to stress, where it protect membranes and proteins against desiccation and oxidative damage. This protection occurs due to its ability to concentrate residual water around proteins (Belton and Gil, 1994) as, due to the flexibility of the bond found in the naturally occurring trehalose dihydrate, the glucose molecules are able to conform to the irregular surface of proteins during hydrogen binding (Paiva and Panek, 1996). When yeast cells accumulate trehalose, they are better able to withstand heat and desiccation stresses (McBride and Ensign, 1987). Plants that accumulate large amounts of trehalose (*e.g.* the resurrection plant *Selaginella lepidophylla*) can survive long periods of near complete desiccation and resume normal growth and development upon rehydration (Adams et al., 1990). Given this myriad of biological functions it is unsurprising that trehalose metabolism has been extensively studied in many organisms. In the next section, I will outline the enzymes involved in its metabolism.

2.6.3. Pathways for trehalose synthesis

Archaebacteria, eubacteria, fungi, plants and animals all possess the ability to synthesize trehalose. Because of this, the evolutionary origin of trehalose is thought to be more ancient than that of sucrose (Paul et al., 2008). Five pathways for trehalose synthesis have been characterized in bacteria, but only one in fungi (with the exception of certain endophytes and mycorrhizal fungi), plants and animals (Figure 2.3) (Avonce et al., 2006). The singular pathway present in fungi, plants and animals is the TPS/TPP pathway, which involves trehalose 6-phosphate (Tre6P) as an intermediate from which trehalose is ultimately derived. The following two pathways are present exclusively in prokaryotes, namely the TreY/TreZ and TreT pathways. The first liberates trehalose from malto-oligosaccharides while the second synthesizes trehalose from ADP-Glc and Glc. The final two can be found in prokaryotes and certain fungi and include the TreS and TreP pathways. The first directly converts maltose to trehalose while the second synthesizes trehalose from Glc and Glc-1P.

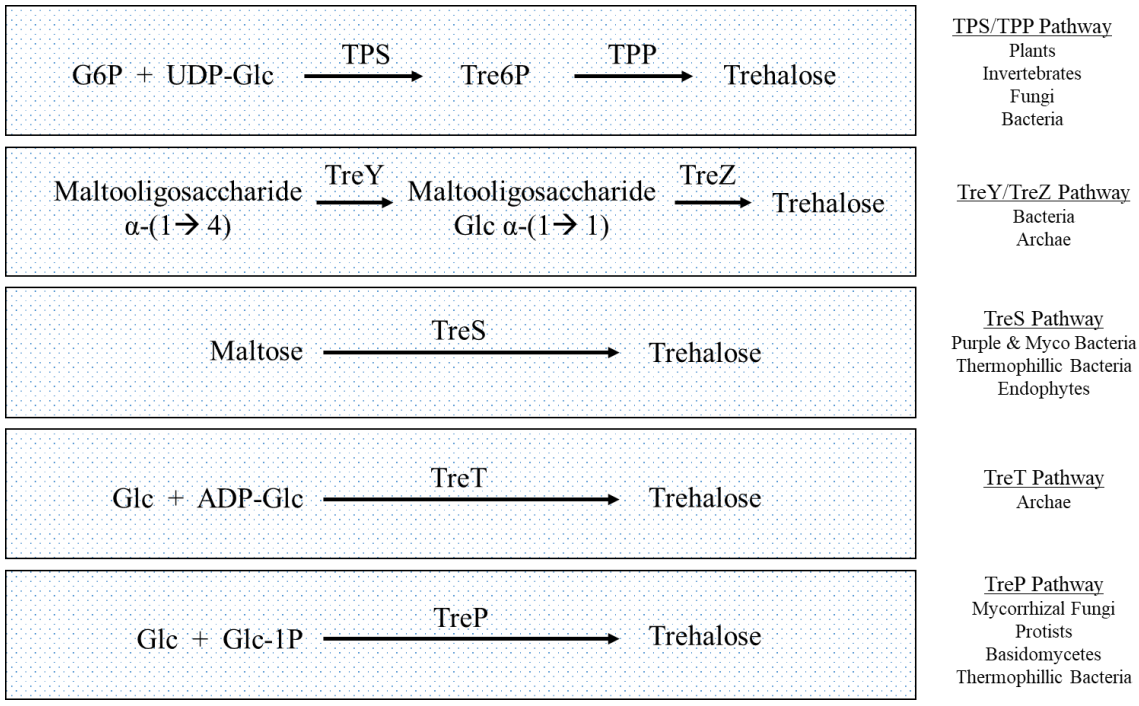

Figure 2.3. Pathways for trehalose synthesis. The organisms the pathways are found in are listed to the right of each reaction. Of the five pathways, the TPS/TPP pathway is the best studied thus far, with the most research carried out in yeast. Acronyms in order of appearance: G6P = glucose 6-phosphate; UDP-Glc = Uridine diphosphate glucose; TPS = trehalose 6-phosphate synthase; Tre6P = trehalose 6-phosphate; TPP = trehalose 6-phosphate phosphatase; TreY = malto-oligosyltrehalose synthase; TreZ = malto-oligosyltrehalose trehalohydrolase; TreS = trehalose synthase; Glc = glucose; ADP-Glc = Adenosine diphosphoglucose; TreT = trehalose glycosyltransferring synthase; Glc-1P = glucose 1-phosphate; TreP = trehalose phosphorylase.

2.6.4. Trehalose metabolism in yeast

Prior to research in plants, trehalose metabolism was studied extensively in *E. coli* and bakers' yeast (*Saccharomyces cerevisiae*). In yeast, several mutants were generated, defective in steps of the pathway, and these have helped greatly in identifying TPS and TPP homologs in plants and other eukaryotes (González et al., 1992, p. 1; O'Hara et al., 2013; Schluepmann et al., 2012). Over the past 35 years, four *tps1* mutants have been characterized (*fdp1*, *cif*, *byp1*, *glc6*), which aided understanding of the role played by trehalose in yeast (Breitenbach-Schmitt et al., 1984; Cannon et al., 1994; Gancedo and Flores, 2004). All of the mutants were generated independently and found to be defective in the same gene, *ScTPS1*. When grown on media supplemented with glucose, these mutants failed to grow (Blázquez and Gancedo, 1994; Navon et al., 1979; van de Poll and Schamhart, 1977) which was found to be due to unregulated glycolysis, where Pi is sequestrated through the synthesis of phosphorylated intermediates with subsequent inhibition of ATP synthesis. Briefly, during glycolysis ATP is consumed in order to drive glucose catabolism, with reserves restored in subsequent metabolic reactions. When a sharp increase in glucose occurs, glycolysis consumes ATP faster than it can be generated, stalling other metabolic processes that need it, leading to "substrate-accelerated death" (Teusink et al., 1998). It was found that growth of *tps1* on glucose can be restored by either decreasing the amount of glucose that enters the cell, or by decreasing the amount that enters glycolysis.

With regards to restricting glucose entry into the cell, it was found that disruption of *CAT3* (*Sucrose NonFermenting 4*, *SNF4*) in the *tps1* background reversed the growth inhibition on glucose (Blázquez and Gancedo, 1994). CAT3 is implicated in regulating the transcription of glucose transporters and, by disrupting the gene, glucose uptake is impaired and this decrease in glucose content restores growth of the *tps1* mutant. Upon entry into glycolysis, glucose becomes phosphorylated, forming Glc6P through the action of hexokinase (HXK). Researchers have investigated the effects Tre6P has on the phosphorylation of fructose and glucose by HXK in various organisms (Blázquez et al., 1993; Blázquez and Gancedo, 1994). In *S. cerevisiae*, Tre6P was found to strongly inhibit phosphorylation by HXKII and weakly by HXKI (Blázquez et al., 1993). Restricting the amount of glucose that can enter glycolysis by disrupting *HXKII* in the *tps1* background, also reversed the growth inhibition observed on glucose media (Hohmann et al., 1993). These results suggest a role for Tre6P as a feedback inhibitor of HXKII (Figure 2.4) (Blázquez et al., 1994). Hence, a disruption in *ScTPS1* would bring about uncontrolled phosphorylation of hexoses, which leads to the depletion of Pi and a

subsequent inability to produce ATP (Blázquez et al., 1994, 1993). These data indicate that trehalose metabolism plays a role as a nutrient sensing pathway in yeast. The discovery of similar pathways in plants led to the concept that trehalose may have a similar function in these organisms and much research in plants has examined it in this context.

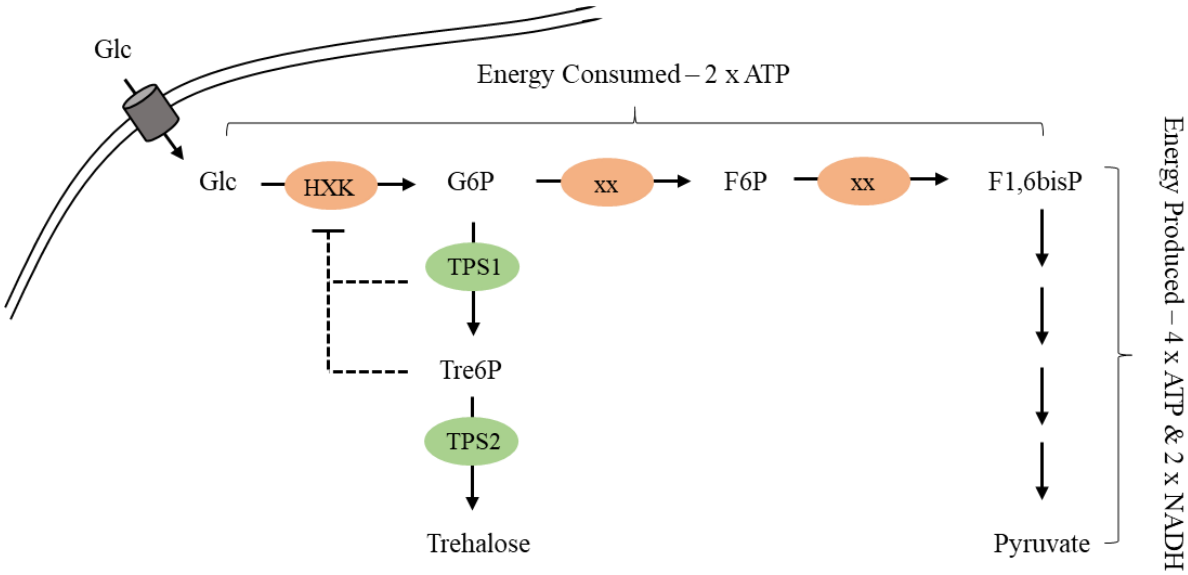

Figure 2.4. Regulation of glycolysis by Tre6P. Glucose enters the cell *via* transporters and in the first committed step of glycolysis, is phosphorylated to Glc6P by HXK. Glc6P is a substrate for Tre6P synthesis, which in turn feedback inhibits HXK. This feedback inhibition regulates the entry of glucose into glycolysis. Also indicated are the energy consuming steps and the energy producing steps. "xx" indicates enzymes involved in glycolysis, not described above. *(Image adapted from Peeters et al., 2017).*

2.6.5. Trehalose metabolism gene families in plants

As mentioned above, plants also make use of the TPS/TPP pathway. In the late nineties, TPS and TPP genes were discovered in *Arabidopsis* through the complementation of yeast mutant strains (Blázquez et al., 1998; Vogel et al., 1998). Since then, genes for trehalose metabolism have been found to be widespread in plants and can be grouped into four gene families, namely TPS Class I and II, TPP and trehalase (TRE, trehalose catabolic enzyme) (Lunn, 2007). The *Arabidopsis*, rice and poplar genomes contain families of 11, 11 and 12 *TPSs* respectively, with phylogenetic analyses differentiating them into two groups (Class I and II) (Leyman et al., 2001). *Arabidopsis* has 4 class I genes (AtTPS1-4), while rice and *Lotus japonica* have 1 and poplar 2 (Leyman et al., 2001; Lunn, 2007). Proteins of both classes contain a

glucosyltransferase-like domain similar to that of ScTPS1 and otsA (*E. coli* TPS), but only the class I proteins are capable of complementing yeast TPS mutants indicating that class II proteins are catalytically inactive. Furthermore, some TPS1 proteins contain an N-terminal extension that appears to be unique to plant TPSs. Upon its removal, the catalytic activity of the enzyme is increased suggesting it is auto-inhibitory (Lunn, 2007). Class II TPSs consists of 10 genes in rice and poplar and 7 in *Arabidopsis* (AtTPS5-11). In addition to the synthase domain, these isoforms contain a region at the C-terminus that is similar to the phosphatase domain in ScTPS2. This domain includes 3 amino acid residues typical of the L-2-haloacid dehalogenase (HAD) superfamily of proteins, suggesting that they may possess TPP activity. However, no such activity has been demonstrated, indicating that the class II TPSs might serve some other function. Furthermore, the active sites within the synthase domain of class II proteins are less well-conserved than in class I TPSs and this is reflected in their inability to complement the yeast *tps1* deletion strain.

The genomes of *Arabidopsis*, rice and poplar all contain 10 *TPP* genes. TPP proteins in *Arabidopsis* also contain the HAD motifs and the residues involved in substrate binding appear to be well conserved (Leyman et al., 2001). Furthermore, these proteins contain a variable N-terminal region of yet undetermined function. When comparing sequences from different species, it appears that gene structure is conserved (Paul et al., 2008). Catalytically active TPP proteins have been identified in numerous plant species, including *Arabidopsis*, maize and rice, *via* complementation of the yeast *tps2* deletion strain (Habibur Rahman Pramanik and Imai, 2005; Satoh-Nagasawa et al., 2006; Vogel et al., 1998). Unlike the two gene families described above, *TRE* is generally encoded by a single gene in all plants studied thus far, with the exception of *P. patens* and poplar, which encode three (Leyman et al., 2001).

When examining the evolutionary origins of these gene families in plants, it has been shown that both classes of TPSs and TRE have a eukaryotic origin, whereas the TPPs have a prokaryotic origin (Paul et al., 2008). Due to similarities in domain structure between the class II TPSs and yeast ScTPS2, it has been proposed that they might have possessed TPP activity at one point in time, which became superfluous with the acquisition of prokaryotic TPPs. As a consequence, class II TPSs may have evolved new functions.

2.6.6. Trehalose metabolism influences growth and development in plants

Due to the autotrophic nature of plants and their tendency to store carbon as either sucrose or starch, it is improbable that trehalose serves a role as either a carbon source or storage carbohydrate (Vogel et al., 2001). A possible regulatory function for the sugar or the proteins involved in its biosynthesis is possible, since plants accumulate only trace amounts of the sugar, yet possess large gene families encoding its biosynthesis (Leyman et al., 2001). When altering the trehalose biosynthetic pathway, phenotypes are observed that suggest the involvement of the pathway (or components thereof) in coordinating plant metabolism and development (Paul et al., 2008). Such phenotypes include alterations in all the major stages of plant development, namely embryogenesis, vegetative development and flowering. The plethora of effects caused by these alterations suggest an important regulatory role for this pathway.

2.6.6.1. Heterologous expression in plants

Prior to the discovery of trehalose biosynthetic genes in plants, researchers tried to engineer the pathway into crop plants to improve drought tolerance. Transgenic *Arabidopsis* and tobacco lines expressing heterologous *TPS* genes presented with increased resistance to abiotic stress and improved photosynthetic rates (Penna, 2003). Furthermore, these plants showed lancet leaf formation and stunted growth. The latter aberrations were later eliminated *via* inducible *TPS* expression (Karim et al., 2007) or the expression of a TPS-TPP fusion protein (Pilon-Smits et al., 1998), while maintaining increased abiotic stress resistance. Conversely, transgenic lines expressing heterologous *TPP* genes showed a reduction in photosynthetic rates while displaying no growth abnormalities (Goddijn et al., 1997, 1997; Holmström et al., 1996; Paul et al., 2001; Penna, 2003; Romero et al., 1997). This improved drought tolerance is unlikely to be due to increased trehalose levels, since it does not accumulate to sufficient levels to serve as an osmoprotectant (Romero et al., 1997), suggesting a more complex role for the sugar in preventing desiccation. These phenotypes were suggested to be caused to changes in carbon allocation, indicating that trehalose (or components of the pathway) might be involved in sugar signaling, affecting several pathways (hormonal and metabolic) that lead to these pleiotropic changes (Goddijn et al., 1997).

2.6.6.2. Further studies point to Tre6P as a key player

A functional *TPS* gene was identified and characterized in *Arabidopsis* using a yeast *tps1*Δ complementation approach (Blázquez et al., 1998; Eastmond et al., 2002). Expression analyses revealed that *AtTPS1* is expressed in all tissues at a low level (Blázquez et al., 1998). An early

study examining homozygous recessive *tps1* mutants demonstrated embryo lethality. Although embryos of *tps1-1* lines were indistinguishable from those of WT in the primary stages of embryo development, upon transition from the heart stage to the torpedo stage, *tps1-1* embryos became progressively retarded, eventually arresting at the latter stage (Eastmond et al., 2002). The heart stage is characterized by cell division and differentiation while the torpedo stage is characterized by cell expansion and storage reserve accumulation. Furthermore, during the switch from the heart to the torpedo stage, an increase in sucrose flux to the embryo is observed, which is needed to support the synthesis of storage reserves and to trigger a cascade of sugar signaling events necessary to bring about this developmental switch (Wobus and Weber, 1999). It would appear that *AtTPS1* plays an important role in responding to the increased sucrose flux, suggesting that disrupting the gene affects sucrose signaling. The growth arrest of the *tps1-1* mutant can be overcome using inducible or tissue-specific expression of *AtTPS1* in the *tps1-1* background; for example by inducing expression during embryogenesis (Dijken et al., 2004) and driving expression using an embryo-specific promoter (*ABI3* promoter) (Gómez et al., 2010, p. 1). After germination however, these plants still show stunted growth and delayed or eliminated flowering suggesting a role for Tre6P in above ground processes. When cultured *in vitro* under normal sucrose concentrations of 340 mM, *tps1-1* embryos arrest at the torpedo stage, however, upon lowering the sucrose concentration to between 30 and 90 mM, cell expansion is significantly enhanced, partially rescuing the phenotype. These findings suggested that components of the trehalose pathway are necessary to ensure normal growth and development.

It was unclear however, whether AtTPS1, Tre6P or trehalose was required for proper embryo development. Trehalose could be excluded since *tps1* embryos cultured on this sugar failed to overcome growth arrest at the torpedo stage (Eastmond et al., 2002; Wingler et al., 2000). These data do not however rule out the embryo lethal phenotype being caused by a second protein that AtTPS1 interacts with, rather than reduces Tre6P. Heterologous expression of *E. coli otsA* in the *Arabidopsis tps1* background (Dijken et al., 2004) using the *AtTPS1* promoter to drive the expression of *otsA* helped to show that the most likely reason for embryo lethality is Tre6P amounts. These plants overcame the embryo lethality and, as otsA is unlikely to interact with other proteins within the host, it indicates that restoration of Tre6P, rather than the presence of AtTPS1, rescues the mutant. This would suggest that Tre6P is essential for the breakdown of supplied sucrose in the developing embryo and that it controls carbohydrate utilization. When assessing the precise role of Tre6P in this regard, researchers first considered

a model resembling that of yeast, whereby AtTPS1 could prevent growth arrest by preventing a metabolic imbalance, brought on by an increased sucrose supply. As mentioned earlier, ScTPS1 and Tre6P regulate the flux of glucose into glycolysis by regulating HXK activity, the primary site for entry into the pathway. However, Tre6P does not inhibit HXK activity in *Arabidopsis*. Yeast mutants defective in HXKII activity are able to overcome *tps1*Δ growth arrest on glucose. However, an *in vivo* approach in the *Arabidopsis tps1-1* background using *AtHXKII* antisense suppression lines, did not restore embryo growth (Eastmond et al., 2002). Furthermore, growth arrest was also not overcome using the HXKII inhibitor, glucosamine. These findings suggested that AtTPS1 and/or Tre6P exert their effects *via* a different mechanism than in yeast.

Further evidence for Tre6P in regulating growth and development came from studies expressing bacterial homologs in plants. Transgenic lines expressing *otsB* (TPP activity) (Schluepmann et al., 2003; Wingler et al., 2012) and *treC* (trehalose 6-phosphate hydrolase; TPH activity) (Schluepmann et al., 2003) had pale green leaves that were increased in size. Since these two sets of plants produced the same phenotype, it was concluded that a depletion of Tre6P was the cause, rather than the formation of the distinct end-products of these two proteins (trehalose for *otsB*; glucose and glucose 6-phosphate for *treC*). This hypothesis was substantiated by Tre6P measurements, where these two transgenic events had a twofold reduction in this metabolite. Transgenic lines expressing *otsA* demonstrated smaller, dark green leaves compared to WT and increased Tre6P content, a phenotype opposite to that of the *otsB* and *treC* expressing lines.

2.6.6.3. The nexus model explains the relationship between sucrose and Tre6P

Monitoring changes in Tre6P content in carbon starved seedlings has started to reveal how this intermediate is implicated in carbon utilization. Such seedlings contained very low levels of Tre6P compared to non-starved counterparts, however, within 15 to 30 min of exogenous sucrose addition, Tre6P amounts rise approximately 25 fold (Lunn et al., 2006). Concurrently, it was found that sucrose levels rose substantially and further experiments demonstrated that Tre6P closely mimicked diurnal sucrose fluctuations in soil-grown *Arabidopsis* rosettes (Carillo et al., 2013; Lunn et al., 2006; Martins et al., 2013). These findings suggested that Tre6P might act as a specific signal for sucrose availability. One framework to understand this would be with regards to the relationship between Tre6P and the constituent molecules (UDP-

Glc and G6P) it is derived from. These hexose phosphates are the breakdown products of sucrose and the amount of Tre6P is related to their concentrations, suggesting that Tre6P influences their accumulation. This helps to explain the observed rapid increase in Tre6P when sucrose is supplied exogenously which might be due to an increased pool of these molecules. In this context, Tre6P reflects the availability of these intermediates and, as an extension, sucrose. All cellular functions can ultimately be derived from these intermediates, and as trehalose and Tre6P are derived from them, they sit at the heart of plant metabolism and development.

2.6.6.4. A link between Tre6P and starch metabolism

Further analysis of the *otsA* and *otsB* over-expressing lines revealed a difference in starch content, with *otsA* lines containing more starch than WT, and *otsB* lines less (Kolbe et al., 2005). These findings implicate Tre6P in the regulation of starch synthesis. In photosynthetic tissues this occurs in chloroplasts, where starch is formed from ADP-Glc by starch synthases and starch branching enzymes, ADP-Glc is synthesised by ADP-Glc pyrophosphorylase (AGPase), an enzyme subject to both redox and allosteric regulation (Hendriks et al., 2003; Stitt and Zeeman, 2012; Tiessen et al., 2002). In *otsA* expressing lines, AGPase is found in a more active, reduced state, whereas in *otsB* lines, it was found in the less active, oxidized state (Kolbe et al., 2005). To verify whether Tre6P modulates the redox state of AGPase, and as an extension stimulates starch synthesis, transgenic lines expressing *otsA* under an ethanol inducible promoter were used (Martins et al., 2013). When inducing expression at the beginning of the day, a rapid rise in Tre6P and only a marginal rise in starch levels were observed at the end of the day. Furthermore, significant changes in the redox state of AGPase were not observed (Martins et al., 2013) and radiolabelling experiments demonstrated that Tre6P did not affect carbon flux into starch (Figueroa et al., 2016). These experiments led researchers to conclude that Tre6P does not modulate the redox state of AGPase and only has a minor effect on starch accumulation during the day. Interestingly, however, it was shown that Tre6P does influence starch breakdown at night (Figueroa et al., 2016; Martins et al., 2013) when transitory starch reserves are mobilized. Ethanol-inducible expression of *otsA* revealed that increased levels of Tre6P at night inhibited starch degradation. It was proposed that Tre6P functions to coordinate starch breakdown with the levels of sucrose in source leaves and sucrose demand from sink tissues (Martins et al., 2013). According to this model, the sucrose-

Tre6P nexus adjusts the rate of starch breakdown to keep the plant from replenishing its reserves before the break of the new day.

2.6.6.5. Tre6P and SnRK1

Growth of heterotrophic sink tissues is dependent on the supply of sucrose from source tissues or the remobilization of starch reserves. When unloaded from the phloem, sucrose is cleaved, and its breakdown products subsequently enter central metabolism. Because it fuels central metabolism in sink tissues, its availability is a significant growth determinant. Two protein kinases play a central role in regulating plant growth by relaying information to the plant regarding environmental conditions and nutrient status (Baena-González et al., 2007; Dobrenel et al., 2016; Emanuelle et al., 2015). One of them, SNF1-related protein kinase 1 (SnRK1), plays a role in energy sensing, homeostasis and abiotic stress responses and, is potentially regulated by Tre6P (Baena-González et al., 2007; Delatte et al., 2011; Zhang et al., 2009). When energy levels are low, SnRK1 is activated which goes about inhibiting processes that consume energy (biosynthetic processes) and stimulating processes that produce energy (catabolic processes). It has been demonstrated that SnRK1 activity is inhibited by Tre6P, Glc6P and Glc1P in crude extracts of developing *Arabidopsis* tissues (Nunes et al., 2013; Zhang et al., 2009). Immuno-precipitated SnRK1 was, however, not inhibited by Tre6P. When the immunoprecipitation supernatant was added to the partially purified protein sensitivity was restored, indicating that other factors are necessary to bring about this inhibition (Zhang et al., 2009).

2.6.7. The elusive class II TPS's

Because the class II TPS proteins are not catalytically active with regards to trehalose metabolism (Harthill et al., 2006; Lunn, 2007; Ramon et al., 2009; Vogel et al., 2001), it has been more difficult to ascertain their role(s) in plant growth and development. They do seem to possess some function, as it has been demonstrated that their expression is spatially and temporally controlled (Avonce et al., 2006; Ramon et al., 2009). In this final section, I will outline what is currently known about these proteins.

2.6.7.1. Extensive transcriptional regulation of Class II TPSs

A comprehensive study examining mRNA accumulation of class II *TPSs* in *Arabidopsis* revealed that they are differentially expressed in a tissue-specific manner and respond differently to nutrient and hormone treatments (Ramon et al., 2009). Nitrate treatment of

hydroponically grown *Arabidopsis* seedlings led to an upregulation in *AtTPS9* and *AtTPS10* (Wang et al., 2003). Sucrose starvation elicited the same response from these two genes as well as *AtTPS8* and *AtTPS11* in *Arabidopsis* suspension cell cultures (Contento et al., 2004; Osuna et al., 2007; Price et al., 2004). Conversely, these four genes were strongly repressed by the addition of sucrose and glucose (Osuna et al., 2007; Price et al., 2004; Ramon et al., 2009; Usadel et al., 2008). *AtTPS5* expression is inversely related to the latter group of genes with regards to sucrose starvation and glucose or sucrose feeding (Usadel et al., 2008). Transcriptional regulation of *AtTPS6* and *AtTPS7* is unaffected by carbon availability (Ramon et al., 2009). Promoter-GUS fusions revealed information regarding the spatial distribution of their expression. Differential expression was observed in certain root apical meristem cell types (Ramon et al., 2009). Furthermore, expression was detected in other actively dividing tissues for several of the genes (Ramon et al., 2009). Expression in meristematic tissues prompted researchers to examine how these genes respond to auxin and cytokinin. Cytokinin strongly upregulated the expression of *AtTPS8* and *AtTPS9* and downregulated the expression of *AtTPS5* while auxin had no effect on any of the genes studied (Brenner et al., 2005; Ramon et al., 2009). Taken together, the differential expression observed with regards to carbon feeding and hormone treatments, suggests a role for these class II TPS proteins in regulating plant growth and morphogenesis by integrating hormone and metabolic signals (Brenner et al., 2005; Contento et al., 2004; Ramon et al., 2009; Thimm et al., 2004). Furthermore, it has been suggested that these genes are not functionally redundant due to the variability in their expression patterns. In summation, it has been proposed that Class II TPSs are involved in sensing Tre6P levels and initiating a sustained (AtTPS5) or rapid (AtTPS8) response (Harthill et al., 2006).

2.6.7.2. Post-translational regulation of class II TPSs

Aside from transcriptional regulation, Class II TPS's are subject to post-translational regulation through phosphorylation and binding of 14-3-3 proteins (Glinski and Weckwerth, 2005; Harthill et al., 2006). 14-3-3 proteins bind target proteins at specific phosphorylated residues, with the association having one of several consequences (Camoni et al., 2018). It can regulate protein activity or affect protein-protein interactions, localization and stability (Finnie et al., 1999; Rolland et al., 2006; Sehnke et al., 2002), which ultimately affect the processes the target proteins are involved in (Camoni et al., 2018). Through 14-3-3 affinity chromatography and other methods, Harthill et al. (2006) demonstrated that 14-3-3 proteins bind to phosphorylated

AtTPS5, AtTPS6 and AtTPS7. Moreover, *in vitro* phosphorylation of several Class II TPSs by SnRK1 were observed through LC-MS/MS (Glinski and Weckwerth, 2005). These TPSs possess SnRK1 phosphorylation sites and, it has been demonstrated that different TPSs are phosphorylated to different degrees (Glinski and Weckwerth, 2005). In summation, it has been proposed that phosphorylation and subsequent 14-3-3 binding of TPS proteins mediate the plants response to signals that activate SnRK1.

2.6.7.3. TPS proteins form complexes

In yeast, the ScTPS1 and ScTPS2 proteins form part of an oligomeric enzyme complex together with the regulatory proteins ScTPS3 and ScTSL1 (Bell et al., 1998; Reinders et al., 1997). ScTPS1 can also exist outside of the complex. This complex catalyses both reactions of the trehalose biosynthetic pathway. Evidence for TPS complexes in plants came from several studies. Fast protein liquid chromatography (FPLC) gel exclusion chromatography of *Arabidopsis* inflorescence crude extracts found AtTPS1 eluting in two fractions of varying size (600-800 kDa and 300 kDa). Both fractions contained the cell cycle kinase CDKA:1 and tubulin (Geelen et al., 2007). Interactions between AtTPS1 and the aforementioned proteins were confirmed *via* yeast two hybrid analyses and co-immunoprecipitations (Geelen et al., 2007). Moreover, in rice, OsTPS proteins interact with themselves and other TPS proteins (Zhang et al., 2011). Their presence in a complex was verified using gel filtration assays which revealed that OsTPS1 co-fractionated with OsTPS5 and OsTPS8 in a 360 kDa complex (Zhang et al., 2011). Researchers have subsequently proposed that TPS complexes could modify Tre6P levels and, in doing so, regulate plant growth and development.

2.6.7.4. Class II TPS's are implicated in stress adaptation

Several Class II TPSs have been implicated in biotic and abiotic stress tolerance in various species. With *AtTPS5* expression is upregulated during heat stress and, removing its activity brings about impaired basal thermotolerance (Suzuki et al., 2008). In cotton, *GhTPS11* expression is upregulated in response to heat, PEG, elevated salt, gibberellic acid and abscisic acid, with downregulation observed in response to sucrose feeding (Wang et al., 2016). Rice *tps8* mutants showed salt and ABA sensitivity, whereas TPS8 overexpressing lines showed improved salt tolerance (Vishal et al., 2019). Furthermore, heterologous expression of *GhTPS11* in *Arabidopsis* led to increased germination inhibition at 4°C, suggesting that GhTPS11 regulates germination during chilling stress (Wang et al., 2016). Regarding biotic stress, *AtTPS11* expression was shown to be upregulated in *Arabidopsis* following *Tobacco*

mosaic virus infection (Golem and Culver, 2003). AtTPS11 is also implicated in defence responses against green peach aphid feeding (GPA) (Singh et al., 2011). Mutant *tps11* lines had an increased number of GPAs on their leaves compared to WT, with resistance restored *via* functional complementation (Singh et al., 2011). Silencing the tomato *SlTPS3*, *SlTPS4* and *SlTPS7* genes reduced resistance to *B. cinerea*, while silencing *SlTPS5* led to increased resistance (Wang et al., 2016). Furthermore, in *Arabidopsis*, *AtTPS5* expression was upregulated in response to *B. cinerea* and *P. syringae* infection, with *tps5* knockout lines showing more susceptibility to infection (Wang et al., 2019). Taken together, it would appear that certain Class II TPSs are implicated in the plants basal defence response against pathogen infection, while others confer tolerance to abiotic stresses. This lack of overlap in function would suggest that different TPSs have specific or specialized functions under different conditions and stresses.

2.6.7.5. More diverse roles for Class II TPSs

Aside from those listed above, Class II proteins are implicated in several other roles. In a study examining mutant lines showing altered phenotypes with regards to cell shape, Chary et al. (2008) identified a mutant called *cell shape phenotype-1* (*csp-1*). This line had a mutation in *AtTPS6*, which led to a loss of pavement cell lobes in the leaf epidermis and indicating a possible role for AtTPS6 in the control of cellular morphology. Knockouts of the other Class II genes had no effect on cell morphology, indicating that AtTPS6 is unique in regulating cell morphology. In the common bean *Phaseolus vulgaris*, *PvTPS9* expression is upregulated in nitrogen-fixing root nodules (Barraza et al., 2016). Silencing *PvTPS9* expression in roots led to structural modifications with regards to cell shape and cell wall thickening, suggesting a role for PvTPS9 in nodule formation and development.

2.7. Conclusion

In summation, although great strides have been made in trehalose and sucrose related research, there is a lack of knowledge remaining related to the enzymes involved in their metabolism and signaling. As it relates to SUS, some of these unknowns include transcriptional regulation and subcellular localization of different SUS isozymes and the differences between the SUS clades. With regards to TPS, the largest unknown is the exact function of class II TPS's.

Aims and Objectives

Rationale

As stated above, the overall aim of the study is to determine whether SUS and class II TPS function has been conserved, or whether new functionality was obtained by Angiosperms since their divergence from the most recent common ancestor. Most research has concentrated on the study of flowering plants as they constitute most crops. There are nevertheless good reasons for the study of similar processes in non-vascular plants. Firstly some bryophytes are used for the production of pharmaceuticals (see Reski et al., 2015 for examples) and so, understanding metabolism in these plants could help optimise their growth. Secondly a more general elucidation of how metabolism has changed during evolution helps in understanding how plants have altered these pathways since they developed vasculature. This may provide insights into the roles they have in influencing plant fitness in the environments where Angiosperms predominate.

The individual aims and objectives were as follows:

Aim 1: Characterization of the *Sucrose Synthase* (*SUS*) gene family in *P. patens* (Chapter 4):

SUS is a key enzyme implicated in phloem loading and sink strength in vascular plants (Stein and Granot, 2019). Non-vascular plants have different morphologies and modes of photosynthate transport, and thus far, nothing is known about the role SUS plays in these basal lineages. Here we aimed to characterize the *PpSUS* gene family in *Physcomitrella*, and to use the results obtained for comparative studies against vascular plants, to infer whether SUS function is similar in the two groups. To this effect, the following objectives were met:

- Identifying putative homologs in the genome of *P. patens*, analysing their gene structures and polypeptide sequences
- Examination of polypeptide sequences to check for the conservation of active site and regulatory residues
- Clarifying their phylogenetic position among *SUS* sequences from multiple land plants
- Examination of their expression during the course of *P. patens* development and over a day/night cycle
- Determination of SUS activity in *P. patens* protein extracts
- Analysis of the sub-cellular localization of PpSUS proteins

Aim 2: Characterization of the class II *Trehalose 6-Phosphate Synthase* (*TPS*) gene family in *P. patens* (Chapter 5):

Very little is known about class II TPS proteins in vascular plants, except for the fact that they are regulated extensively at the transcriptional level (Ramon et al., 2009). Bryophytes share some characteristics with extant land plants and may provide some clues as to the original function of the class II proteins. Here we aimed to characterize the class II *PpTPS* gene family in *Physcomitrella*, to clarify whether vascular plants possibly evolved new functions for class II proteins. To this effect, the following objectives were met:

- Identifying putative homologs in the genome of *P. patens*, analysing their gene structures and polypeptide sequences
- Clarifying their phylogenetic position among *TPS* sequences from multiple land plants
- Analysis of their expression during *P. patens* development, in response to stress and hormone treatments and, over a day/night cycle
- Examination of PpTPS polypeptide sequences to check for the conservation of residues implicated in TPS and TPP catalytic activity
- Determining whether class II PpTPS proteins demonstrate TPS or TPP catalytic activity
- Revealing the sub-cellular localization of PpTPS proteins
- Determining whether PpTPS proteins interact with each other

Using these approaches, our current understanding of SUS and TPS function have been broadened in non-vascular plants.

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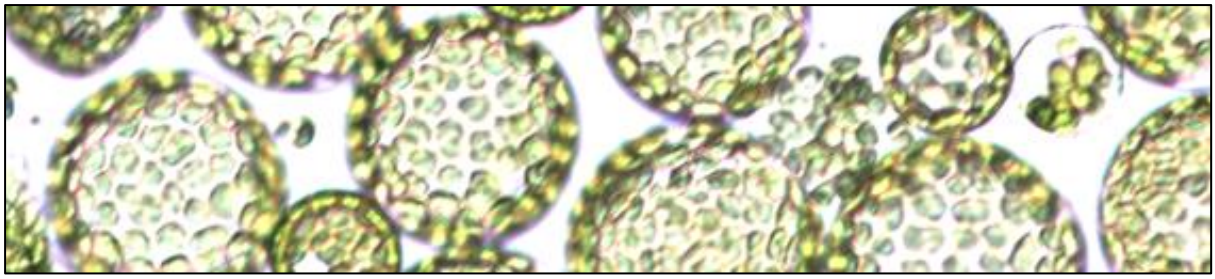

Chapter 3

Materials and Methods

3.1 Chemicals

Chemicals and kits were obtained from Sigma (Steinheim, Germany), Roche (Manheim, Germany) and Thermo Scientific (Waltham, United States) unless stated otherwise. All primers were obtained from Inqaba Biotec (Johannesburg, South Africa) and are listed in Table 3.1. All antibodies (primary and secondary) used in this study were obtained from Thermo Scientific.

3.2. Plant material and culture conditions

For this study the Gransden ecotype (Engel, 1968) of *Physcomitrella patens* was used as a wild type (WT). Plants were cultured using a protocol adapted from Collier and Hughes, (1982) and Cove et al. (2009). Briefly, protonemal tissue was sub-cultured on BCD-agar plates (0.7% (w/v) agar, 1 mM $CaCl_2$, 45 µM $FeSO_4 \cdot 7H_2O$, 1 mM $MgSO_4$, 1.84 mM KH_2PO_4, 10 mM KNO_3, trace elements, 5 mM ammonium tartrate) overlaid with cellophane discs and grown in a growth room at 25^0C, under a 16h/8h light/dark photoperiod using cool white fluorescent lights (50 µM photons m^{-2} s^{-1}; OSRAM L 58W/640, Germany).

3.3. Identification of homologs, phylogenetic tree construction and sequence analyses

Putative *P. patens* TPS and SUS isoforms were identified by a TBLASTN search against the *P. patens* genome in Phytozome (https://phytozome.jgi.doe.gov) using the *A. thaliana* AtSUS1 (NP_001332603.1) or the AtTPS1 (NP_001322932.1) protein sequences. Sequences from other species were collected from either Phytozome *v3.3* or the NCBI (https://ncbi.nlm.nih.gov) database. Protein alignments of conserved domains were performed in CLC workbench (CLC Bio; https://www.qiagenbioinformatics.com).

Sequences were aligned using MUSCLE (Edgar, 2004) in MEGA X (Kumar et al., 2018; version X; https://www.megasoftware.net) and manually examined for correction where necessary. For the *TPS* phylogenetic tree, the GTR+G substitution model was used, while for the *SUS* tree, the GTR+G+I substitution model was used. Both models with the lowest Bayesian Information Criterion scores were used. These scores were considered to best describe the substitution model. For each model, corrected Aikake Information Criterion values, Maximum Likelihood values and the number of parameters were calculated (Nei and Kumar, 2000). To estimate the ML values, a tree topology was automatically computed, with the analysis involving 52 (*TPS*) and 49 (*SUS*) nucleotide sequences. Codon positions included 1st, 2nd and 3rd non-coding. In total there were 2054 (*TPS*) and 2048 (*SUS*) positions in the final dataset. Finally, evolutionary analyses were conducted in MEGA X (Kumar et al., 2018), using the Maximum Likelihood method and the General Time Reversible model (Nei and Kumar, 2000). The tree with the highest log likelihood (-70373.61 = *TPS*; -68517.48 = *SUS*) is shown, with the percentage of trees within which the associated taxa clustered, shown next to the branches. Initial trees for the heuristic search were obtained by applying the Maximum Parsimony method. A discrete Gamma distribution was used to model evolutionary rate differences among sites, with 5 categories (+*G*, parameter = 1.1707 (*TPS*) and 1.1493 (*SUS*)). The tree was drawn to scale with branch lengths measured in the number of substitutions per site. Final trees were edited in InkScape 0.92 (https://inkscape.org/). Outgroups for each tree included a *TPS* sequence from *E. coli* (*otsA*) and a *SUS* sequence from *N. punctiforme* (*NpSUS*).

3.4. RNA Isolation and cDNA synthesis

One hundred milligrams of *P. patens* protonemal material was homogenized in liquid nitrogen using a mortar and pestle. This was used for RNA isolation using the Qiagen RNeasy Plant Mini Kit according to the manufacturer's recommendations. One microgram of total RNA was DNase treated using the RNAse free Dnase I system (Thermo Scientific) and used for first strand cDNA synthesis using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturers recommendations.

3.5. RT-qPCR

Primers were designed to amplify segments of the coding sequence of the *SUS* and *TPS* homologs as well as *Actin 5* (reference gene; Pp3c10_17070; Le Bail et al., 2013) using the PrimerQuest Tool. Amplicon sizes were set to 100 bp and primer pairs examined for specificity

using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) using *P. patens* (taxid:3218) as the reference genome. RT-qPCR reactions were carried out using the PowerUp™ SYBR Green Master Mix (Thermo Fisher Scientific). A reaction volume of 12 µl contained 2 µl cDNA (representing 50 ng cDNA), 0.25 µl of each 0.8 µM primer and 6 µl Mastermix with the entire reaction brought to volume by the addition of nuclease free water. The QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific) was used to conduct the reaction using the machines default cycling parameters. Three biological repeats and 3 technical repeats per biological repeat were used with the standard curve being drawn up from seven serial dilution points using 25 ng as the most concentrated dilution of cDNA.

To calculate relative fold change, the threshold cycle number (ΔCt) was used in the ΔΔCt method, with the "middle of the day" (M.O.D) sample used as a calibrator (Livak and Schmittgen, 2001). This experiment was conducted in compliance with the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" (Bustin et al., 2009).

3.6. *in Silico* analysis of gene expression

For *in silico* expression analysis of *P. patens* development, data were obtained from the *Physcomitrella* EFP browser (http://bar.utoronto.ca/efp_physcomitrella/). Deposited data were from two array experiments (NimbleGen v1.6 *P.patens* 135k arrays) where the Gransden WT ecotype was grown under controlled conditions at 25°C under a 16h/8h light/dark photoperiod with 50% humidity. For the induction of gametangia and sporophyte development, growth conditions were changed to a short day regime at 17°C (Ortiz-Ramírez et al., 2016). For *in silico* expression analysis of *P. patens* hormone and stress treatments, data were obtained from GeneVestigator (https://genevestigator.com), deposited by Perroud et al. (2018). Briefly, mRNA used for cDNA synthesis and subsequent global deep sequencing, were from protonemal tissue cultured on BCD plates supplemented with acetone or 1 µM GR24 (strigolactone) dissolved in acetone; gametophores cultured above liquid Knop media supplemented with 10 µM IAA (auxin) or without (control); gametophores cultured on BCD media moved to empty petri dishes for 180 h (dehydrated) and rehydrated on sterile water for 5 min following which they were moved to BCD plates for 2 h prior; protonema cultured on BCD media subjected to heat stress treatments (5 h at 22ºC, 1 h at 37ºC) for 5 days.

3.7. Protein localization

Full length CDS's lacking stop codons were amplified from protonemal or rhizoid cDNA by PCR using Q5® High-Fidelity DNA Polymerase and cloned into the pENTR/D-TOPO™ entry vectors. Primers used for amplification can be found in Table 3.1. Sense orientation was confirmed *via* PCR and entry clones sequenced prior to use in LR Clonase II reactions for the creation of pMP1383 expression constructs. In these vectors, the CDSs were cloned in frame with a C-terminal GFP fluorophore. Prior to protoplast transformation, 60 µg of each construct was linearized by digestion with 20 units of *SfiI* (Thermo Scientific) at 50°C overnight. DNA was column purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's recommendations, before transformation.

Seven-day old protonemal tissue was used to isolate protoplasts according to Cove et al. (2009). In short, protonemal tissue was digested in a 2% (w/v) Driselase solution in 8.5 % (w/v) mannitol, with gentle shaking at room temperature for 1 h. Subsequently, the solution was centrifuged at 250 *g* for 5 min. The resulting pellet was washed three times in 8% (w/v) mannitol and quantified using a haemocytometer to determine protoplast number. Finally, the pellet was re-suspended in MMM (5 mM MES-NaOH pH 5.6; 8.5% (w/v) mannitol; 5 mM $MgCl_2$) solution to a final concentration of 4.6 million protoplasts per mL. Once resuspended in MMM solution, the protoplasts were incubated at room temperature (RT) for 20 min prior to transformation. Protoplasts were transformed according to Lui and Valdi (2011). Briefly, 600 µl of the protoplast suspension was mixed with 700 µl PEG/Ca solution (7% (w/v) Mannitol; 100 mM $Ca(NO_3)_2$; 10 mM Tris-HCl (pH 7.2); 40% (w/v) PEG-4000) and 60 µg linearized plasmid DNA in a 10 mL culture tube. The mixture was incubated at RT for 30 min and 6.5 mL of liquid BCD solution containing 8.5% (w/v) mannitol added dropwise. Transformants were incubated in the dark overnight to allow sedimentation.

After a 16 h incubation period in the dark, transformed protoplasts were used to visualize GFP emissions using a Zeiss LSM 780 Confocal microscope with an Alpha Plan-Apochromat 100x/1.46 oil DIC M27 Elyra objective. The two lasers used were the 488 laser at 6% (emission wavelength 490-569) and the 405 laser at 1.8% (emission wavelength 668-735). The master gain was set to 800 for the 488 and 600 for the 405 lasers, respectively. Beam splitters used were the MBS 488/561/633 for the 488 laser and MBS 405 for the 405 laser. Images were captured with a 2.0 zoom with Z-stacks taken every 1.842 µm.

3.8. Isolation of total protein from *P. patens*

Homogenized material obtained from section 3.4 was used for protein isolation. For enzyme assays, 100 mg material was re-suspended in ice-cold 500 µl extraction buffer (50 mM MOPS-NaOH (pH 7.2), 1 mM EDTA, 2.5 mM DTT, 5 mM $MgCl_2$, 1mM PSMF, EDTA-free cOmplete™ protease inhibitor cocktail) and vortexed briefly. The homogenate was centrifuged for 10 min at 18 000 *g* at 4°C, and the supernatant desalted using Amicon Ultra 3 kDa centrifugal units (www.merckmillipore.com) according to the manufacturer's recommendations. Desalted extracts were quantified using the Bio-Rad Protein Assay Dye Reagent (www.biorad.com) according to the manufactures recommendations, an assay based on the Bradford method (Bradford, 1976) and kept on ice until their use in assays.

3.9. Sucrose synthase and invertase activity assays

For the SUS assay in the synthesis direction, 50 µl of the desalted extract was added to 250 µl of the reaction mixture (50 mM Tris-HCl (pH 8.4), 5 mM $MgCl_2$, 1 mM PEP, 0.2 mM NADH, 15 mM fructose, 2 mM UDP-glucose, 2 U/ml pyruvate kinase, 10 U/ml lactate dehydrogenase) at 30°C. Decrease in absorbance was determined at 340 nm. The liberation of UDP was coupled to the oxidation of NADH in the presence of the two enzymes. The decrease in A_{340} was measured spectrophotometrically and used to calculate the number of sucrose equivalents synthesised. For the alkaline INV reaction, 10 µl of the desalted extract was added to 90 µl of the reaction mixture (100 mM MOPS-NaOH (pH 7.5), 200 mM sucrose) and incubated at room temperature for 3 hs. Reactions were terminated at 95°C for 5 min and assayed for glucose. To this end, 10 µl of the reaction was added to 250 µl assay buffer (50 mM Tris-HCl (pH 6.9), 5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD, 1 U/ml Glucose 6-phosphate dehydrogenase). Reactions were initiated by adding 1U/ml hexokinase and the increase in A_{340} measured. The change in OD_{340} was used to calculate glucose equivalents. For the acid INV reaction, 10 µl of the desalted extract was added to 90 µl of the reaction mixture (Na-acetate (pH 4.7), 100 mM sucrose) and incubated at 37°C for 1 h. Reactions were terminated at 95°C for 5 min and assayed for glucose as before. Controls for each assay consisted of boiled protein extracts incubated in the same reaction mixtures under the same conditions.

3.10. Yeast complementation

Full length CDS's lacking stop codons were amplified from *P. patens* protonemal cDNA and *M. polymorpha* thallus cDNA using Q5® High-Fidelity DNA Polymerase and restriction cloned into a yeast multicopy pYX212 vector (gift of Professor Patrick van Dijk from the Laboratory of Molecular Cell Biology, KU Leuven, Belgium) as follows: amplicons and the vector were column purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's recommendations and digested with the appropriate restriction enzymes (*EcoRI* and *XmaI* for *PpTPSA, C* and *D*; *EcoRI* and *NcoI* for *PpTPSB*) for 3 h at 37°C. Digested amplicons were resolved on a 1% agarose gel and subsequently purified using the same kit. Amplicons and the linearized vector were combined in an Eppendorf tube and used in a ligation reaction using T4 DNA Ligase (Thermo Scientific, USA) according the manufacturers recommendations. The ligation reaction resulted in amplicons ligated in frame with a HA (human influenza hemagglutinin) tag (Delorge et al., 2015) at the 3' end. Constructs were sequenced prior to use in yeast transformation. For complementation, the *Saccharomyces cerevisiae* W303-1A WT strain (*Matα leu2-3, 112 ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100 GAL SUC2*), the *tps1Δ* deletion strain (YSH290; W303–1A, *tps1Δ::TRP1*), the *tps2Δ* deletion strain (YSH448; W303–1A, *tps2Δ::HIS3*) and the *tps1Δtps2Δ* deletion strain (YSH567; W303-1A *tps1Δ::TRP1, tps2Δ::LEU2*; Hohmann et al., 1993) were used. Control plasmids included an empty vector, the *pYX212*::ScTPS1 for the *tps1Δ* and *tps1Δtps2Δ* strains and the *pYX212*::ScTPS2 for the *tps2Δ* strain. Yeast was transformed according to Gietz and Woods (2002). For drop assays, mutant and WT transformed cultures were grown overnight at 30°C in synthetic complete medium lacking uracil (SC-ura) containing 2% (v/v) galactose. Overnight cultures were diluted into fresh medium and grown to an OD_{600} of 0.5. Once the latter OD_{600} was reached, transformants were serially diluted six times. Four microliters of each dilution were spotted onto SC-ura containing 2% (w/v) glucose or galactose and grown at either 28°C (control) or 38.6°C for 48 h and growth scored after 48h.

3.11. Yeast two-hybrid assay

Entry clones generated in Section 3.7, and those generated in the same way for the class I genes, were used in LR Clonase II reactions to generate bait (pDEST32) and prey (pDEST22) expression plasmids supplied by the ProQuest Two-Hybrid System. Plasmids were transformed into the supplied MaV203 (MATα, leu2-3,112, *trp*1-901, *his*3Δ200, *ade2*-101, *gal*4Δ, *gal*80Δ, *SPAL*10::*URA*3, *GAL*1::l*acZ*, *HIS3*$_{\text{UAS GAL1}}$::*HIS3@LYS2*, *can*1^{R}, *cyh*2^{R}) yeast

strain according to the manual. Transformed yeast strains were inoculated in SC-leu/-trp medium and grown overnight at 30°C in a shaking incubator. These were diluted to OD_{600} of 0.5 and a 1:16 dilution spotted on selection plates. These included SC-leu/-trp/-ura, SC-leu/-trp/+0.2% 5FOA, SC-leu/-trp/-his + 100 mM 3AT and YPAD overlain with a nitrocellulose membrane for X-Gal staining. YPAD plates were grown overnight at 30°C, the membranes frozen in liquid N_2 and used for X-Gal staining according to the Invitrogen Manual. The other selection plates were incubated for 2 days and the growth scored.

3.12. Generation of protein expression constructs

Entry clones used for localization experiments (section 3.6), were incubated with LR Clonase II and pDEST24 to recombine genes in frame with a GST (glutathione S-transferase) tag at the 3' end. To generate a pDEST24::GST tag-only control vector, empty pENTR/D-TOPO constructs were used in the LR Clonase II reaction.

3.13. Screening for active SUS isoforms in *E. coli*

The expression plasmids were transformed into *E. coli* BL21 (DE3) chemically competent cells and colonies containing plasmids re-suspended in M9 minimal medium to an OD_{600} of 0.5. These were serially diluted and spotted onto M9 minimal medium plates (33.7 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.55 mM NaCl, 9.35 mM NH_4Cl, 1 mM $MgSO_4$, 0.3 mM $CaCl_2$, 1µg/L biotin, 1µg/L thiamine and trace elements (13.4 mM EDTA, 3.1 mM $FeCl_3$-$6H_2O$, 0.62 mM $ZnCl_2$, 76 µM $CuCl_2$-$2H_2O$, 42 µM $CoCl_2$-$2H_2O$, 162 µM H_3BO_3, 8.1 µM $MnCl_2$-$4H_20$)) containing either 0.5% (w/v) glucose or 0.5% (w/v) sucrose and supplemented with 0.5 mM IPTG and 100 µg/ml ampicillin. Plates were incubated at 37°C and 28°C for two days after which growth was scored.

3.14. Total protein isolation from *E. coli*

Total protein was isolated from *E. coli* cultures using a protocol adapted from Datta et al. (2015). Briefly, expression vectors were transformed into the BL21 (DE3) *E. coli* strain and a single colony inoculated in 5 ml Lysogeny Broth (LB; 10 g/L peptone, 10 g/L NaCl, 5 g/L yeast extract, pH 7.0) containing ampicillin at 100µg/ml and grown with shaking at 37°C overnight. One millilitre of overnight culture was inoculated into 100 ml of LB-Amp^{100} and grown under the same conditions until an OD_{600} of 0.6 was reached. At that point IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to a final concentration of 0.3mM and the

cultures incubated as before for a further 4 h. Cells were sedimented at 2 500 *g* for 30 min and pellets washed twice in 50 ml 1X PBS at pH7.4. Pellets were subsequently re-suspended in 10 ml extraction buffer (1x PBS pH 7.4, 1 mM DTT, 1 mM PMSF, 1 mM EDTA pH 8.0 and EDTA-free cOmplete^{TM} protease inhibitor cocktail) and lysozyme (Sigma-Aldrich) added to a concentration of 1mg/ml and the suspension incubated on ice for 30 min with occasional mixing. Triton X-100 and DNase I were added to the suspension at 0.2% (v/v) and 5µg/ml respectively. Tubes were shaken vigorously and incubated for a further 1 h at 4°C with gentle shaking. Solutions were centrifuged at 18 000 *g* for 10 min at 4°C and the supernatant clarified by passing through a 0.45 µm syringe.

3.15. Total protein isolation from *S. cerevisiae*

Colonies of yeast strains transformed with complementation constructs (section 3.10) were inoculated into 5 ml SC-ura + 2% glucose medium and allowed to grow overnight at 28°C with constant shaking. Starter cultures were subsequently used to inoculate 50 ml SC-ura + 2% glucose medium and grown until an OD_{600} of 1 as before. Cells were harvested *via* centrifugation at 250 *g* for 5 min at 4°C. Pellets were washed twice in 30 ml 1x PBS (pH 7.4) and finally re-suspended in 400 µl ice-cold lysis buffer (50 mM Tris-HCl pH7.4, 10% glycerol, 2.5 mM $MgCl_2$, 0.2 M NaCl, 1% Igepal (NP-40)). Glass beads were added, and the solution transferred to 2 ml tubes for fast prepping. Cells were fast prepped thrice with 1 min incubations on ice in between. Lysates were centrifuged at 18 000 *g* for 10 min at 4°C before supernatants were transferred to fresh tubes.

3.16. Immunoblot analysis of heterologous protein expression in *E. coli* and *S. cerevisiae*

Protein concentration was determined using the Bio-Rad Protein Assay Dye Reagent (www.biorad.com) according to the manufacture's recommendations, an assay based on the Bradford method (Bradford, 1976). Forty micrograms of crude protein extract were separated using SDS-PAGE (4-12% gradient) and subsequently transferred to a nitrocellulose membrane. Membranes were probed for heterologous proteins using HRP (horseradish peroxidase)-coupled anti-GST high affinity goat antibodies (*E. coli*) or HRP-coupled anti-HA (hemagglutinin) high affinity goat antibodies (*S. cerevisiae*). Chemiluminescence was visualized using luminol and peroxide as substrates (www.agrisera.com) within a LAS-3000 imaging system (www.fujifilm.com).

Table 3.1. Primers used. Accessions for each gene are given to the left of gene names.

Gateway Cloning

Locus Name	Gene Name	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5' - 3')	Size (bp)
Pp3c5_17730	*PpTPS1*	CACCATGCCGTACTCTCTGACAAT	TCATAAATCATGCCTAGGGA	3150
Pp3c6_16450	*PpTPS2*	CACCATGTATGAGGGAGGGCGTAT	GGGTCGCTGCCATCGGTCAGTTGA	3096
Pp3c5_6990	*PpTPS3*	CACCATGGCGGAAGGGGAGAGC	GATGTGCAAAGACGTCTCTAAGG	2568
Pp3c7_15250	*PpTPS4*	CACCATGATGTCACGGTCGTATAG	TATGAAGAGATGAGAAATCAGAAAGC	2673
Pp3c11_17560	*PpTPS5*	CACCATGGTTTCAAGGTCGTAT	AATATGAAGGGATGAGAAATC	2673
Pp3c16_19150	*PpTPS6*	CACCATGGCGGAAGGGGAGAGC	GATGTGCAAAGATGTCTCCA	2559
Pp3c5_19770	*PpSUS1*	CACCATGGATGGTATTGCGACCCA	CACTTTCTTTTCTTGTTCT	2475
Pp3c1_33460	*PpSUS2*	CACCATGGATGGTATTGCGACCCA	CACTTTCTTTTCTTGTTCT	2508
Pp3c19_5850	*PpSUS3*	CACCATGTCGCAGCCACGGCCGA	TGTATGACCAGTAACAGCT	2535
Pp3c10_11330	*PpSUS4*	CACCATGGCGGCAAACGGAGTGG	GTGTATAGCAGTAGATTCCA	2571

Restriction Cloning

Locus Name	Gene Name	Primer Names with Restriction Sites	Primer Sequences (5' - 3')	Size (bp)
Pp3c5_6990	*PpTPS3*	pptps3 *EcoRI* Fwd	GGAATTCATGGCGGAAGGGGAGAGC	2568
		pptps3 *XmaI* Rev	TCCCCCGGGGATGTGCAAAGACGTCTCT	
Pp3c7_15250	*PpTPS4*	pptps4 *EcoRI* Fwd	GGAATTCATGATGTCACGGTCGTATAGC	2673
		pptps4 *NcoI* Rev	CATGCCATGGCATGAATATGAAGAGATGAGAAATC	
Pp3c11_17560	*PpTPS5*	pptps5 *EcoRI* Fwd	GGAATTCATGGTTTCAAGGTCGTAT	2673
		pptps5 *XmaI* Rev	TCCCCCGGGTGAGGCATTTGCCAACCC	
Pp3c16_19150	*PpTPS6*	pptps6 *EcoRI* Fwd	GGAATTCATGGCGGAAGGGGAGAGC	2559
		pptps6 *XmaI* Rev	TCCCCCGGGGATGTGCAAAGATGTCTCCA	

RT-qPCR

Locus Name	Gene Name	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5' - 3')	Size (bp)
Pp3c5_17730	*PpTPS1*	CGAAACAAGGTGGATTGGAT	AGAAACACTGGAACGCAACC	104
Pp3c6_16450	*PpTPS2*	CCTACCCCGTCTGTCACATC	ACTTTTCGCGTCAACCTCAT	101
Pp3c5_6990	*PpTPS3*	AGACTTCGAACGGACCTTATTG	TGATGGACGCTTGTGGTATC	102
Pp3c7_15250	*PpTPS4*	GTGCATTGGTGTGGCATTATC	GGCTCATTGGCTAGTACACTT	105
Pp3c11_17560	*PpTPS5*	TCGATTATGCGAGGCACTTT	CTGAGCGGCCATAATACTCTAC	100
Pp3c16_19150	*PpTPS6*	CAAATCAACCCGTGTCTGTTAAG	CCATCATCGCCAGTAGTTCTT	103
Pp3c5_19770	*PpSUS1*	CGTTCTAGGAATGCCCGATAC	GTCCAGTCCCTGTAGCTTTATG	106
Pp3c1_33460	*PpSUS2*	GGCACGTCTCGACAAAGTAA	CTCCTCCAACAATGACCAAGT	101
Pp3c19_5850	*PpSUS3*	TCAATTCCTTCGCCTCCATAC	GGCCTCTTCAGCTCTAACTAAC	106
Pp3c10_11330	*PpSUS4*	CCAAAGAAGCCCGTCTTACA	GTTTGACGTATCGGGAGAGAAG	109
Pp3c10_17070	*PpActin5*	CCGTCGAGCATGAAGATCAA	ATCTGTTGGAACGTGCTGAG	102

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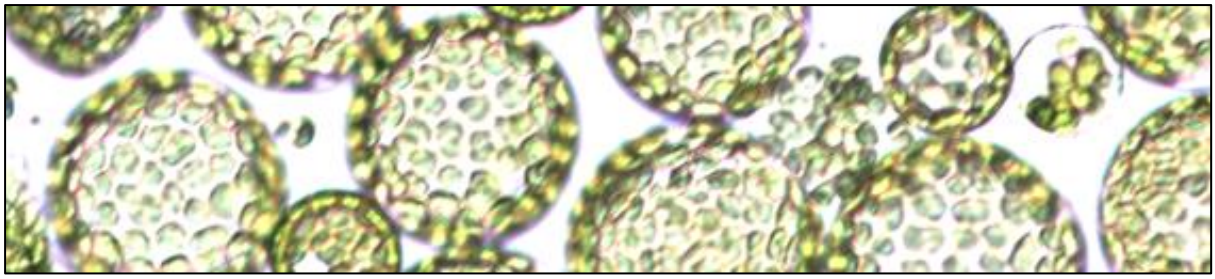

Chapter 4

Characterization of the *Sucrose Synthase* (*SUS*) gene family in *Physcomitrella patens*

Anna J. Wiese, Ethel E. Phiri, Jens Kossmann, David Honys, James R. Lloyd

Author Contributions

The first author (AJW) performed all of the experimental work within the chapter (with the exception of the phylogenetic tree) and wrote the dissertation. With regards to the tree, AJW collected all the sequences and performed initial alignments, while EEP curated the alignment and constructed the tree. JRL conceived of, and supervised the study, while JK and DH provided resources. This chapter is partially written in a style aimed at publication.

4.1. Abstract

Sucrose is central to plant growth and development, being the main end-product of photosynthesis, which is translocated from source to sink tissues. Concentrations of this sugar need to be balanced between these tissues to ensure optimal growth. The main route of carbon entry from sucrose into central metabolism is believed to be *via* sucrose synthase (SUS), an enzyme implicated in phloem loading and sink strength. Research related to the function of SUS in non-vascular plants is lacking, and in this study I report the characterization of the *SUS* gene family in *P. patens*. A search against the *Physcomitrella* genome revealed 4 putative *SUS* genes. Phylogenetic analysis differentiated *SUS* sequences into 5 clades, with *PpSUS* sequences falling within a bryophyte-specific clade. Amino acid sequence alignments demonstrated the conservation of residues involved in SUS activity. The latter was demonstrated using activity analyses of *P. patens* crude protein extracts. Furthermore, expression analyses indicate overlapping and unique expression patterns for the four genes as it relates to developmental stage expression and over a day/night cycle. Finally, localization experiments revealed that all PpSUS proteins are cytosolic. This study provides novel insights into the evolution of *SUS* genes in *P. patens* and, will serve as a platform for the design of future experiments related to this gene family in non-vascular plants.

4.2. Introduction

Sucrose plays a fundamental role in plant growth and development, as it is the main end-product of photosynthesis translocated *via* the phloem in many plants. A balance in sucrose contents between source and sink tissues needs to be maintained to ensure optimal plant growth. Only four enzyme types mediate sucrose synthesis and cleavage (SPS, SPP, INV, SUS) with spatial and temporal coordination of their activities creating a system whereby sucrose is effectively transported between source and sink tissues. This balance is maintained by various sugar signaling pathways that seek to balance sucrose synthesis and consumption in order to avoid energy stress.

The primary route of sucrose breakdown in vascular plant heterotrophic tissues is believed to be *via* SUS (Chey and Nelson, 1976; Counce and Gravois, 2006; Craig et al., 1999; Zrenner et al., 1995), although exceptions do exist (Angeles-Núñez and Tiessen, 2010; Barratt et al., 2009; Bieniawska et al., 2007; Chengappa et al., 1999). It is highly regulated at several levels and is implicated in determining both phloem loading and sink strength (Stein and Granot, 2019; Wang et al., 1993). Furthermore, the subcellular localization of isoforms can be cytosolic or plasma membrane associated, where they are thought to participate in the synthesis of cellulose (Amor et al., 1995). Sucrose synthases are generally encoded by small multigene families. It has been proposed (Volpicella et al., 2016) that different isoforms have the same functions, but are active either in distinct cell types, during different developmental stages, or under different environmental conditions. A recent study examining the localization of SUS isoforms in *Arabidopsis*, identified that members of each clade were found in the same tissues (Yao et al., 2020), lending some confidence to the hypothesis put forward by Volpicella et al. (2016). Despite extensive research efforts in vascular plants, various aspects of SUS remain elusive. For example, how *SUS* expression is regulated, how the localization of isozymes are regulated, the difference between the SUS clades, why SUS and INV co-evolved and, how sucrose cleavage is divided between the two enzyme groups (Stein and Granot, 2019).

Research related to carbon metabolism in bryophytes are still at its infancy. Anatomically, bryophytes share certain similarities with vascular plants, as water and nutrient conducting cells are present in the gametophyte (Ligrone et al., 2000; Sakakibara et al., 2003). Experiments over the past 40 years have revealed a symplasmic mode of transport within gametophyte tissues where, plasmodesmata form a conduit for transport (Raven, 2003; Regmi et al., 2017; Reinhart and Thomas, 1981). Feeding experiments using ^{14}C-labelled sucrose in the moss *Polytrichum communne* demonstrated the existence of organized long-distance photosynthate

transport, pointing to source-sink relationships akin to those in vascular plants (Reinhart and Thomas., 1981). Labelled sucrose was observed moving from mature phyllids (leaf-like structures) to heterotrophic tissues, or tissues with high mitotic activity. Furthermore, sucrose transporters (SUTs) and SUS have been immunolocalized in the gametophyte-sporophyte interface in *Physcomitrella*, demonstrating that sucrose is actively transported from the gametophyte to the nutritionally dependent sporophyte and metabolized (Regmi et al., 2017). In the desiccation tolerant moss *Bryum argenteum*, a transcript encoding *SUS* was found to be highly upregulated 2 hs post dehydration suggesting the involvement of the protein in its ability to tolerate water loss (Gao et al., 2017).

Aside from these studies, very little research has been carried out on SUS in bryophytes and, because they differ greatly from vascular plants, various open questions remain. For example, how do these plants utilize sucrose cleaving enzymes compared to vascular plants; do these enzymes have functions unique to bryophytes; do they fall within the well characterized clades? Do they present with the dynamic localization patterns observed in vascular plants? How are they regulated and, how do they function together with INV to balance out supply and demand throughout the plant? To address the gap in research related to SUS proteins in bryophytes, I chose *P. patens* as our model to characterize the small multigene family encoded within its genome. Here I report the characterization of four *SUS* genes, with future directives for research discussed in Chapter 6.

4.3. Results

4.3.1. Identification of *SUS* homologs in *P. patens*

As a first step in characterizing the *SUS* gene family in *P. patens*, putative orthologs were identified using a tBLASTn search conducted in the Phytozome v3.3 database using the AtSUS1 protein sequence (NP_001332603.1) as a query. Sequences recovered represented four putative *SUS* genes, classified hence forth as *PpSUS1-4*. The coding sequences of these ranged between 2478 and 2574 bp. The predicted polypeptides encoded at the four loci ranged in size between 826 and 857 amino acids (aa), demonstrating a percentage identity to AtSUS1 of between 60-62% while the similarity between the PpSUS proteins ranged from 65- 84%.

Table 4.1. *Physcomitrella patens SUS* homologs revealed through tBLASTn. Accessions from Phytozome are included to the right of the gene name I ascribed each locus. The max score describes the quality of the alignment, the query coverage the percentage overlap between the query and the subject, the E value the significance of the score and finally the percentage identity indicates the percentage similarity between the two amino acid sequences.

Gene	Phytozome Accession	Max Score	Query Coverage	E Value	Identity
PpSUS1	Pp3c519770	1040	100%	0	60.67%
PpSUS2	Pp3c133460	1046	99%	0	61.57%
PpSUS3	Pp3c195850	1008	100%	0	60.37%
PpSUS4	Pp3c1011330	1029	100%	0	60.17%

4.3.2. Sequence analysis of *Physcomitrella* SUS homologs

Intron exon boundaries were examined within the genomic sequences. As shown in Figure 4.1.A, *PpSUS2* and *PpSUS4* have 12 introns while *PpSUS3* has 13 and *PpSUS1* 14. Sucrose synthases proteins can have one of two architectures, where the gene encodes either a solitary sucrose synthase domain, or both a glycosyl transferase and a sucrose synthase domain (Jayashree et al., 2008). All PpSUS proteins adopt the latter structure, containing both an N-terminal sucrose synthase domain and a C-terminal glycosyl transferase domain (Figure 4.1.B). These domains are of similar size across all four proteins with the sucrose synthase domain being about 545 aa residues and the glycosyltransferase domain about 165 aa residues (Figure 4.1.B).

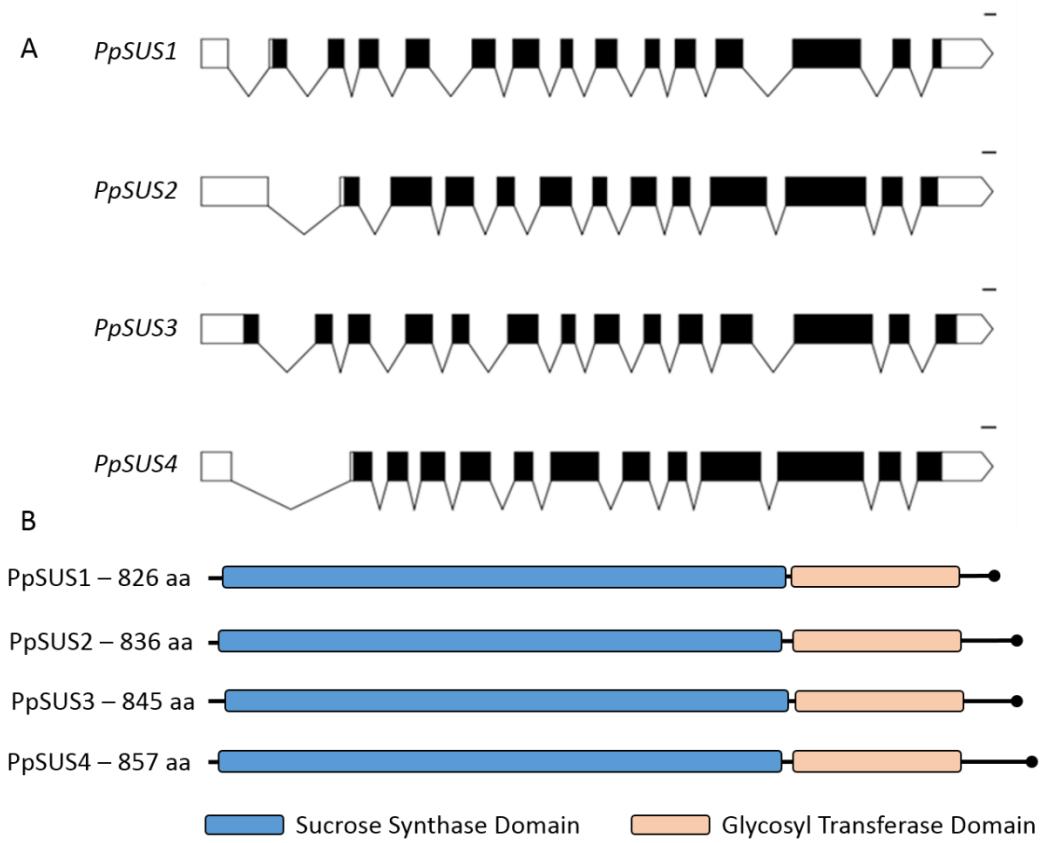

Figure 4.1. Sequence analyses of the PpSUS family. (A) Gene structures of all four *SUS* genes. Scale bars represent 100 bp. White boxes indicate untranslated regions, black boxes indicate exons and lines introns. (B) PpSUS proteins possess both sucrose synthase and glycosyltransferase domains. The length of the proteins are indicated to the right of the names and the circle to the right of the illustrations indicate the C-termini.

Alignment of the sucrose synthase domains reveals good conservation across the majority of sites, with ~60% of overall sites being identical (Figure 4.2). Furthermore, two serine residues (ser-15 and ser-170), known to be phosphorylated in angiosperm SUS's are conserved in all four proteins (Zheng et al., 2011). However cysteine-266, a site known to be important for SUS regulation *via* thiolation (Röhrig et al., 2004; Zheng et al., 2011), is replaced by aspartic acid (C266D). Residues for fructose binding (glycine-302,303 and histidine-287; Zheng et al., 2011) are conserved. Finally, histidine-438, a residue necessary for UDP-Glc binding (Zheng et al., 2011), is also conserved in all of the PpSUS proteins.

Figure 4.2. Sequence alignment of *Physcomitrella* SUS sucrose synthase domains. Protein sequences were aligned in CLC workbench, with the level of conservation indicated in the bar chart below the alignment. Residues are coloured according to the RasMol amino acid colour scheme.

Alignment of the glycosyltransferase domains of the four PpSUS proteins revealed complete conservation of the sites necessary for nucleotide sugar binding (Winter and Huber, 2000; Zheng et al., 2011) while other residues involved in UDP binding (arginine, lysine and glutamic acid; black arrows; Figure 4.3; Sawitri et al., 2018) are also conserved. The latter residues have been shown to be conserved within a variety of glycosyltransferase domains from various enzymes (*e.g.* SPS and glycogen synthase; Sawitri et al., 2018).

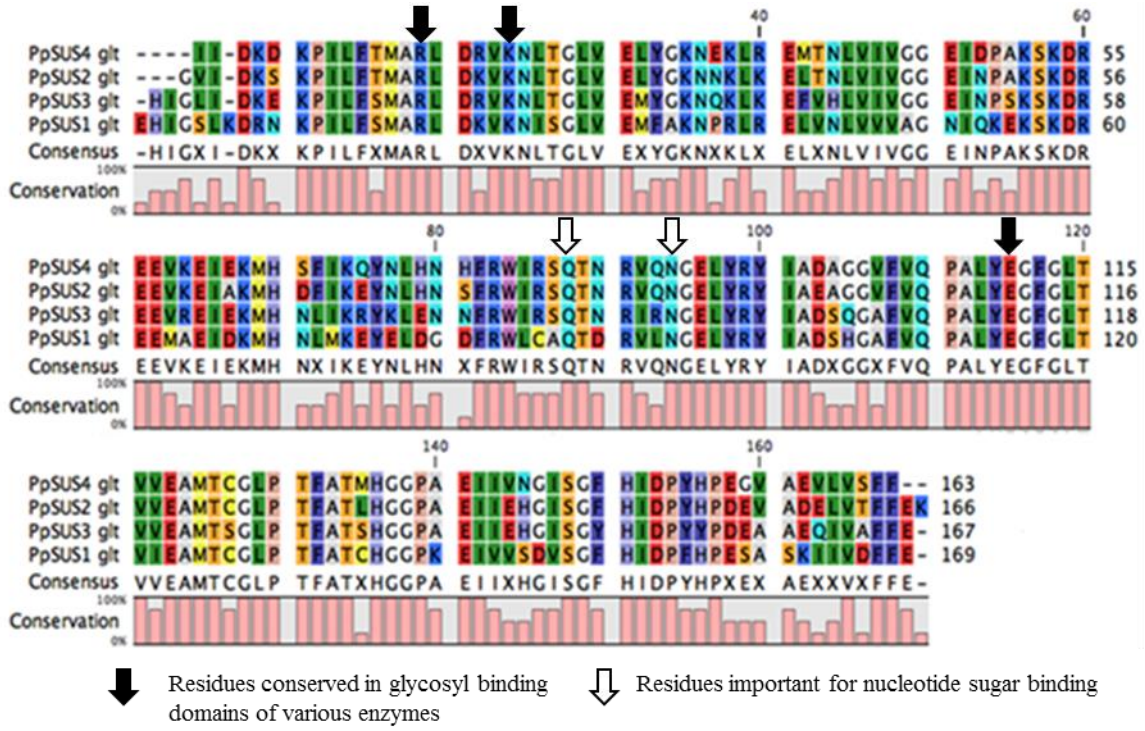

Figure 4.3. Sequence alignment of *Physcomitrella* SUS glycosyltransferase domains. Protein sequences were aligned in CLC workbench, with the level of conservation indicated in the bar chart below the alignment. Residues are coloured according to the RasMol amino acid colour scheme.

4.3.3. Phylogenetic analysis of plant *SUS* genes

Phylogenetic analyses are known to differentiate *SUS* genes into three different clades (reviewed in Stein and Granot, 2019). A comprehensive phylogenetic tree was created using sequences obtained from species ranging from cyanobacteria to angiosperms (Figure 4.4). Species included green algae (*Monoraphidium neglectum, Auxenochlorella protothecoides, Coccomyxa subellipsoidea, Chlamydomonas reinhardtii, Volvox carteri, Chlorella variabilis*), bryophytes (*Marchantia polymorpha, Physcomitrella patens*), lycophytes (*Selaginella moellendorffii*) gymnosperms (*Picea glauca, Picea sitchensis*) and angiosperms (*Arabidopsis thaliana, Solanum lycopersicum, Oryza sativa, Populus trichocarpa, Amborella trichocarpa*). A *SUS* sequence from the cyanobacterium *Nostoc punctiforme*, was used to root the tree. Coding sequences were collected from Phytozome and NCBI and aligned using MUSCLE (Edgar, 2004). Evolutionary history was inferred using the Maximum Likelihood method and the Time Reversible model (Nei and Kumar., 2000), and a maximum likelihood tree constructed.

As expected, angiosperm *SUS* sequences differentiated into three well characterized clades (Stein and Granot, 2019). Sequences from gymnosperms, bryophytes and algae differentiated into 3 other clades, creating a total of 6. Within the angiosperm clades, monocot and dicot sequences were further differentiated into two sub-groups within clade I. Furthermore, *A. trichopoda*, which represents the most extant angiosperm lineage, had two solitary *SUS* sequences within clades II and III. Sequences from the two *Picea* spp., fell within a gymnosperm specific clade, however, the solitary *SUS* sequence from the lycophyte *S. moellendorrffii*, did not group together with either gymnosperms or bryophytes. Within the bryophyte clade, *PpSUS1* appears to be most closely related to *SUS* from the liverwort *M. polymorpha*, than to the remaining three *P. patens* sequences.

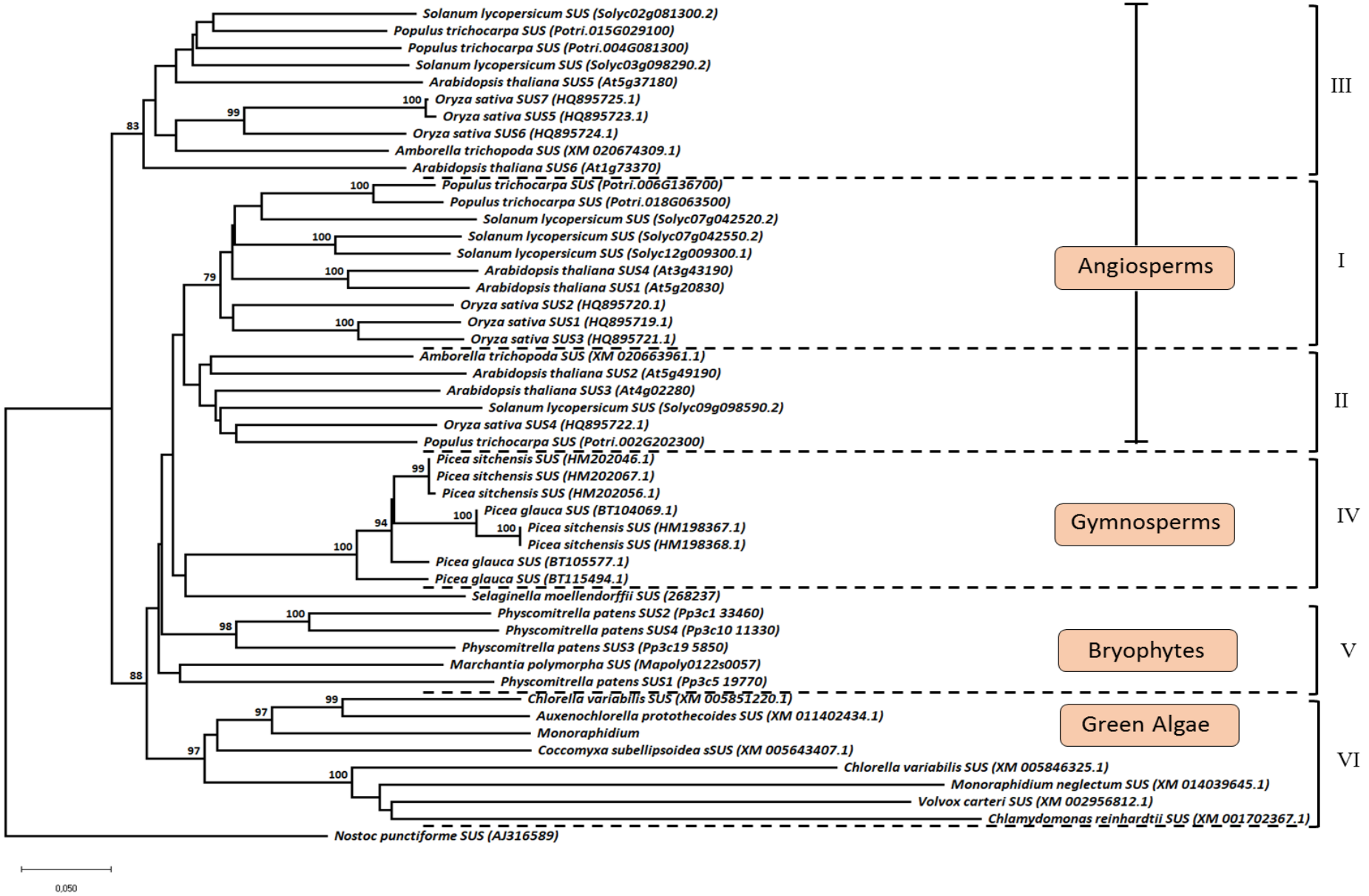

Figure 4.4. Phylogenetic classification of *SUS* sequences from 16 species. Evolutionary history was inferred using the Maximum Likelihood method and Time Reversible model (Nei and Kumar., 2001). The tree with the highest log likelihood (-68517.48) is shown. There were a total of 2048 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). Values displayed at nodes are bootstrap values indicating the phylogenetic confidence of the given node, with bootstrap values below 75% not shown.

4.3.4. *PpSUS* expression analyses

in silico expression analyses described the expression profiles for each *PpSUS* gene both during development and in different tissues. Publicly available microarray data were used to extract information related to these genes for 2 developmental stages (gametophyte and sporophyte) and 11 different tissue types (Figure 4.5; Ortiz-Ramírez et al., 2016). Highest expression throughout development was observed for *PpSUS2*, which showed greatest expression in archegonia and lowest in spores. An increase in expression was observed during sporophyte developmental stages 1 and 2, followed by a drop as the tissue matures. Highest expression in archegonia was also observed for *PpSUS1*, with similar levels in gametophores. Lowest expression was observed in spores, similar again to *PpSUS2*. Very low levels of expression was observed for *PpSUS3*, with mature sporophyte enriched expression, with a 5 fold increase in expression compared to the average expression of the other stages. Finally, *PpSUS4* had the lowest level of expression for all genes, with a slight increase in expression in rhizoids.

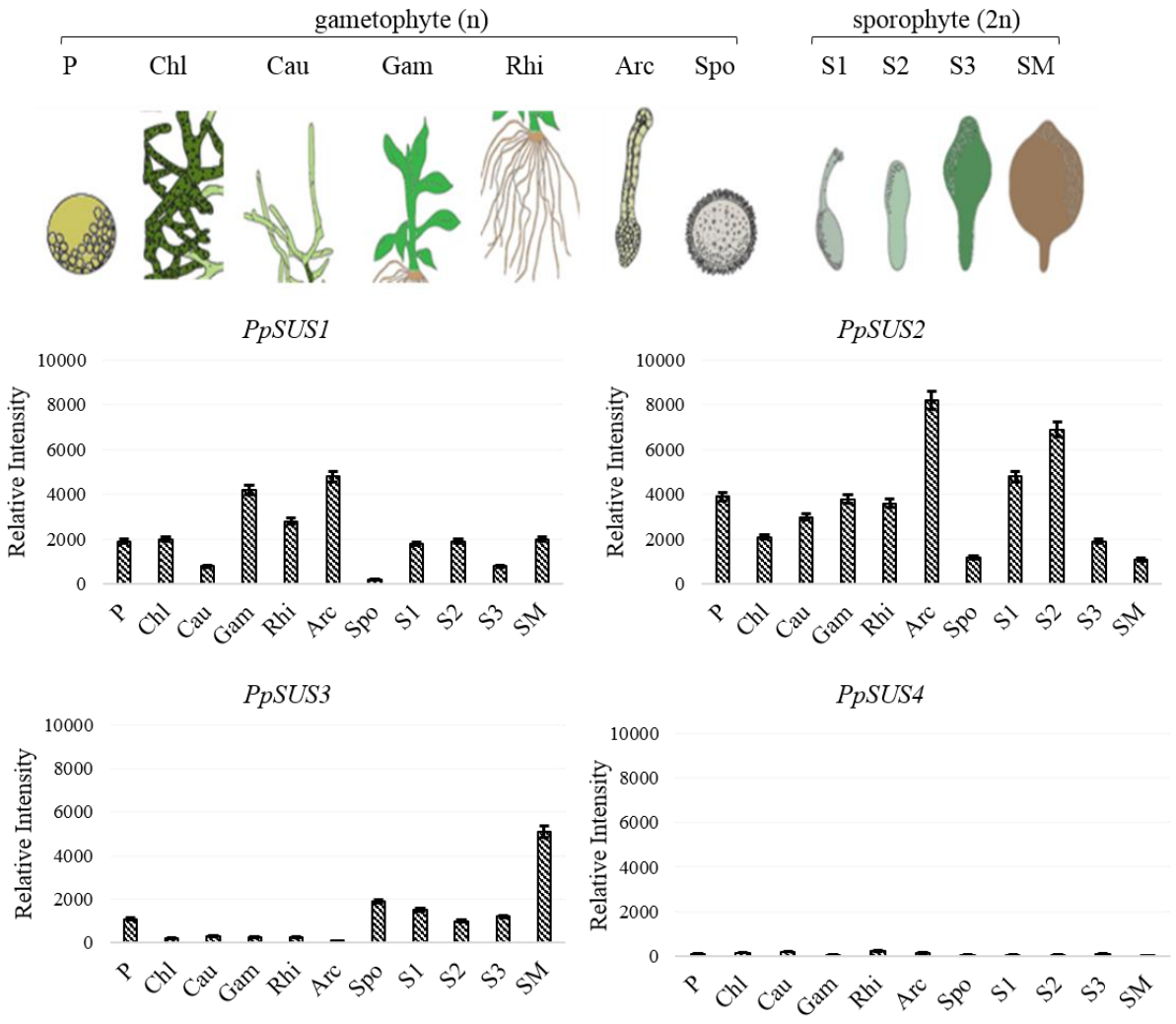

Figure 4.5. *in silico* expression analyses of *PpSUS* gene expression in different developmental tissues. Data were obtained from eFP browser which is a repository of publically available transcriptomic data sets. Data are from Ortiz-Ramírez et al. (2016). Acronyms include: P = protoplast; Chl = chloronema; Cau = caulonema; Gam = gametophyte; Rhi = rhizoids; Arc = archegonia; Spo = spore; S1 – S3 = different stages of sporophyte development; SM = mature sporophyte.

Real-time quantitative PCR (qPCR) was used to examine changes in expression in whole moss colonies (gametophores and protonema) over a day/night cycle. Normalization was performed using *PpActin5* expression and calibration using middle of the day (16:00) as the calibrator sample. Results are reported as normalized fold change (Figure 4.6). Transcript levels for *PpSUS2*, *PpSUS3* and *PpSUS4* showed a similar expression pattern throughout the cycle, however, *PpSUS2* showed a much larger increase in expression at the end of day time point (08:00). In the case of *PpSUS1*, a steady increase in expression was observed nearing the middle of the night, with a large increase in expression at the end of the night (a 3 fold increase compared to 04:00).

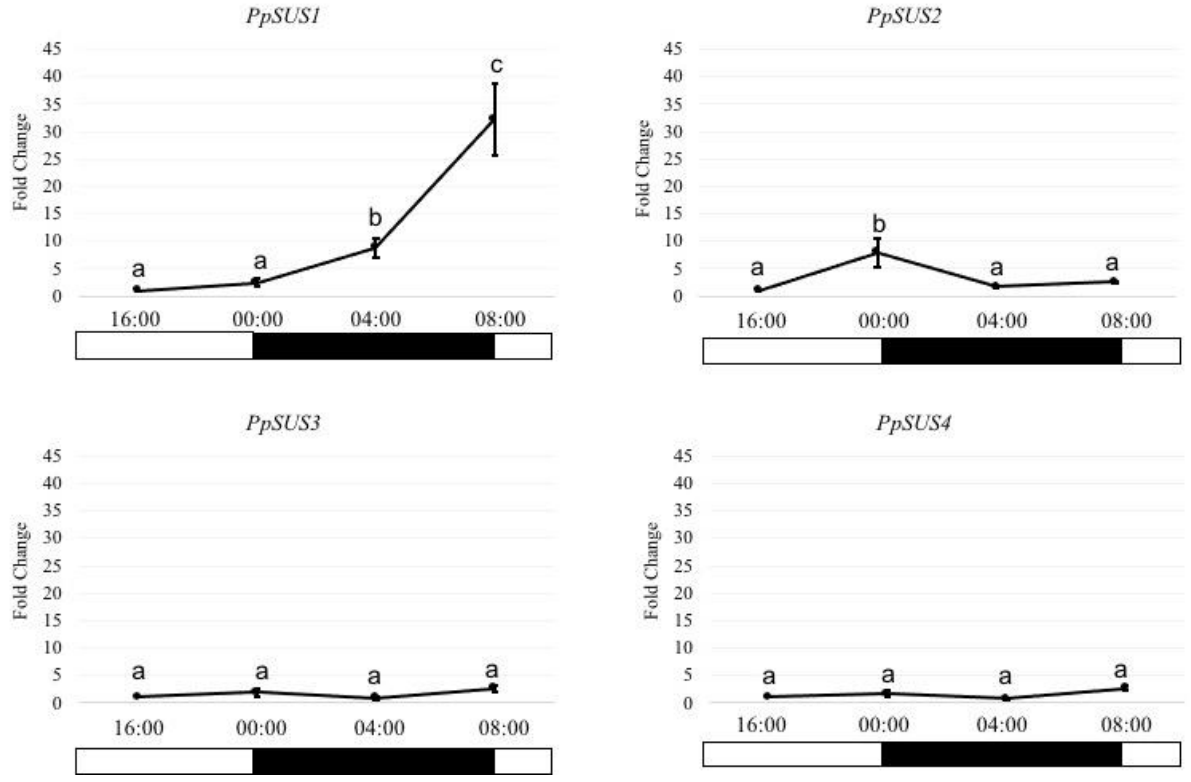

Figure 4.6. qRT-PCR expression of *PpSUS* genes over a day/night cycle. Six week old whole moss colonies were collected at the four time points indicated, with one colony representing one biological replicate. Expression analyses of *PpSUS1-4* during a day/night cycle using qPCR. Data represent normalized fold change of *PpSUS* relative to the reference gene *Actin 5* (Pp3c10_17070), using the time point 16:00 as the calibrator sample. Values represent the mean ± standard error of three independent biological replicates calculated from the Ct values using the ΔΔCt method (Livak and Schmittgen, 2001). Error bars represent standard errors, and when not visible, are within the circular symbols. Significant differences are indicated by different letters, as determined by a Bonferroni-Holm *post hoc* test following a one way ANOVA. A value of 1 represents no expression deficiency (*i.e.* alterations in expression compared to the calibrator sample, 16:00). Black boxes beneath the time points on the x-axes indicate the dark periods and white boxes, the light periods.

4.3.5. Sucrose Synthase and Invertase activity measurements over a day/night cycle

To ascertain whether *P. patens* possesses SUS activity and whether it correlates with the qPCR expression analyses, I extracted protein for activity assays from the same samples as in section 4.3.4. In addition to SUS assays, I conducted both neutral and acid invertase (INV) assays. Crude protein extracts were desalted and used for assays in the synthesis direction for SUS and in the degradative reaction for neutral and acid INV. Sucrose synthase activity showed an increase towards the end of the day, remaining high throughout the night (Figure 4.7). Activities of both invertases were highest during the middle of the day, with a decrease in activity taking place nearing the end of the night period (Figure 4.8).

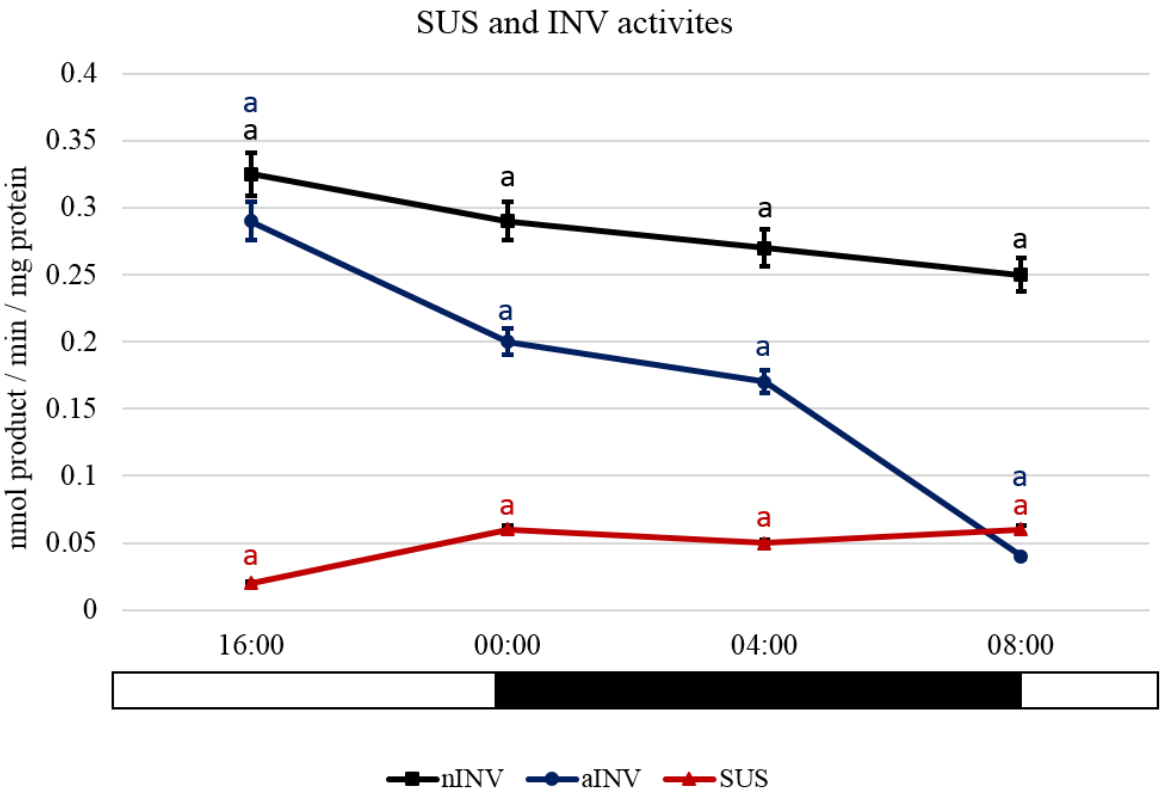

Figure 4.7. SUS and INV activities over a day night/cycle. Six week old whole moss colonies were collected at the four time points indicated, with one colony representing one biological replicate. SUS activity in the synthetic direction was measured over the four time points using desalted crude protein extracts incubated with 15 mM Frc and 2 mM UDP-Glc as substrates in optimal buffer conditions and, the amount of UDP liberated measured enzymatically. INV activity was measured over the four time points using desalted crude protein extracts incubated with 100 mM sucrose as a substrate in optimal buffer conditions for either acid INV (aINV) or neutral INV (nINV). The liberated glucose was measured enzymatically. Data represents mean ± standard error of three independent biological replicates. Error bars represent standard errors, and when not visible, are within the different symbols. Significant differences are indicated by different letters, as determined by a Bonferroni-Holm *post hoc* test following a one way ANOVA. Black boxes beneath the x-axes indicate the dark periods and white boxes, the light periods.

4.3.6. Screening for PpSUS activity in *E. coli*

To determine which orthologs encode catalytically active proteins, I transformed *E. coli* with constructs carrying *PpSUS* genes. Many lab strains of *E. coli* are unable to grow when sucrose is supplied as the sole carbon source (Jahreis et al., 2002), despite the ability to transport it from the media *via* a permease (Sahin-Tóth et al., 1995). By transforming such an *E. coli* strain with constructs carrying the *PpSUS* genes, it should enable its growth on media containing sucrose as the sole carbon source and would indicate that the gene encodes an active SUS. To this effect, CDSs lacking stop codons were cloned into the pDEST24 expression vector containing the inducible T7 promoter, in frame with a glutathione s-transferase (GST) tag for confirmation of heterologous expression *via* immunoblotting. The negative control constituted an empty vector expressing GST alone. The vectors were introduced into *E. coli* BL21 (DE3) and overnight cultures were serially diluted and spotted onto plates containing M9 minimal medium supplemented with either 0.5% (w/v) glucose or 0.5% (w/v) sucrose and incubated at either 30°C or 37°C for 48 hs. Although all strains grew on the glucose plates, I observed no growth on sucrose plates at either temperature (Figure 4.9.A). To understand why, I sought to check whether the proteins are expressed in *E. coli*. To this effect, colonies were inoculated into liquid M9 medium containing 0.5% (w/v) glucose supplemented with 0.5 mM ITPG. Protein was extracted from the cultures and used for immunoblot analysis to determine whether heterologous proteins were expressed. As demonstrated in Figure 4.9.B, PpSUS-GST proteins seem to be present as signal is only found in the strains containing the expression vectors. Although all appear to contain full length polypeptides based on the predicted sizes of the fusion proteins, there are also many bands of lower molecular weight, indicating that much of the protein was degraded.

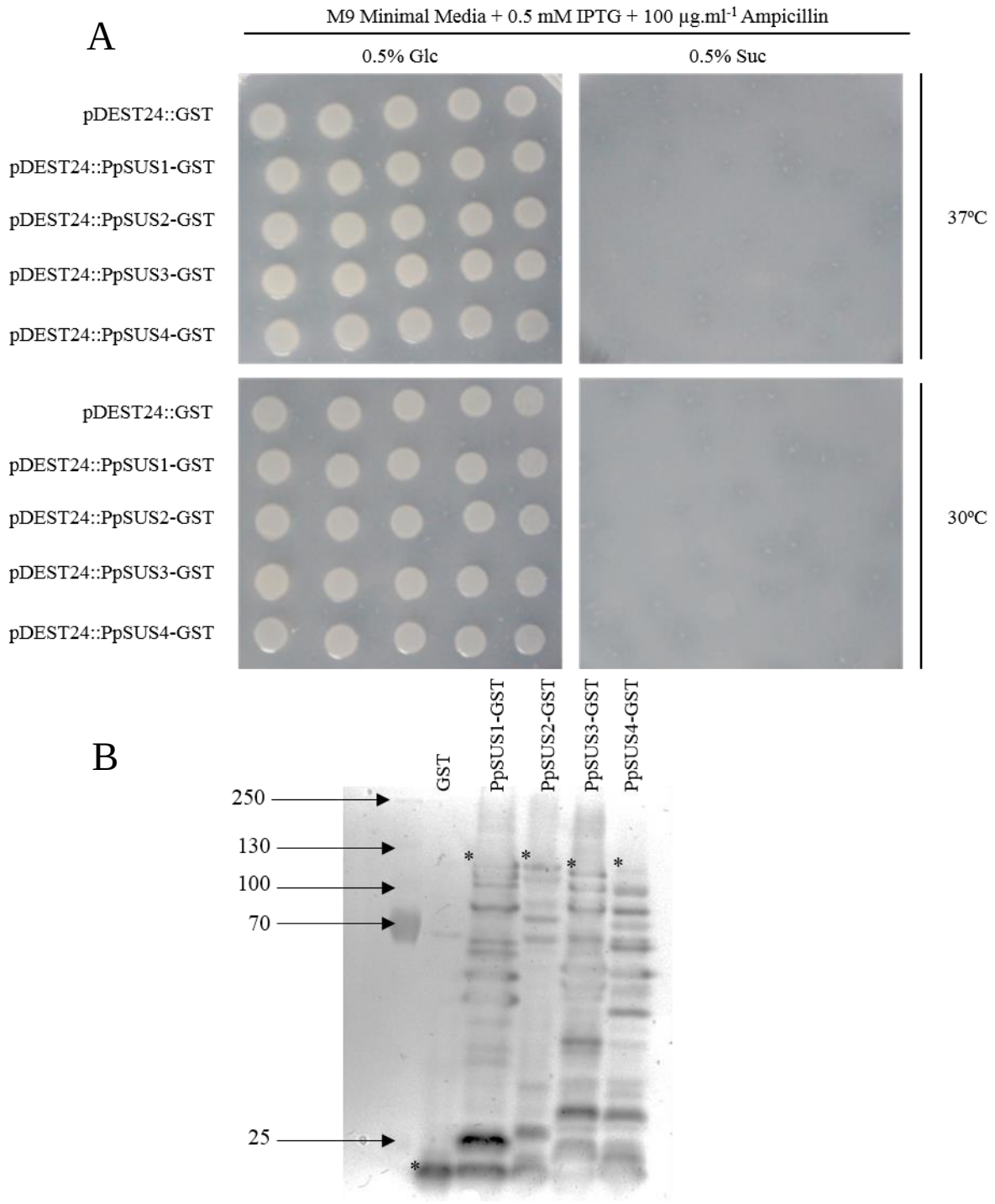

Figure 4.8. Growth of *E. coli* BL21 (DE3) transformed with constructs carrying *P. patens SUS* homologs. (A) Constructs encoding PpSUS-GST fusions were introduced into BL21 and grown on M9 minimal media supplemented with 0.5% (w/v) glucose or 0.5% (w/v) sucrose and growth scored. An empty plasmid served as the negative control. (B) Immunoblot analysis to determine expression of SUS proteins in BL21 (DE3). Proteins sizes are as follows: GST = 26.76 kDa, PpSUS1-GST = 122.95 kDa, PpSUS2-GST = 123.13 kDa, PpSUS3-GST = 124.69 kDa and PpSUS4-GST = 125.61 kDa. Asterisks indicate full length fusion proteins.

4.3.7. *Physcomitrella* SUS homologs localize to the cytosol.

Sucrose synthases demonstrate dynamic localization patterns, with phosphorylation influencing this (Winter et al., 1998). To examine the subcellular localization of the four PpSUS proteins, coding sequences lacking stop codons were ligated in frame with the green fluorescent protein (GFP) under the control of the constitutive 35S promoter for transient expression in *Physcomitrella* protoplasts. Untransformed protoplasts served as a negative control. After a 24 h dark incubation period, GFP emissions were detected using a confocal microscope and images captured. No GFP fluorescence was observed for the untransformed protoplasts while in transformed protoplasts GFP fluorescence can be observed only in the space surrounding chloroplasts, indicating that all proteins are cytosolic.

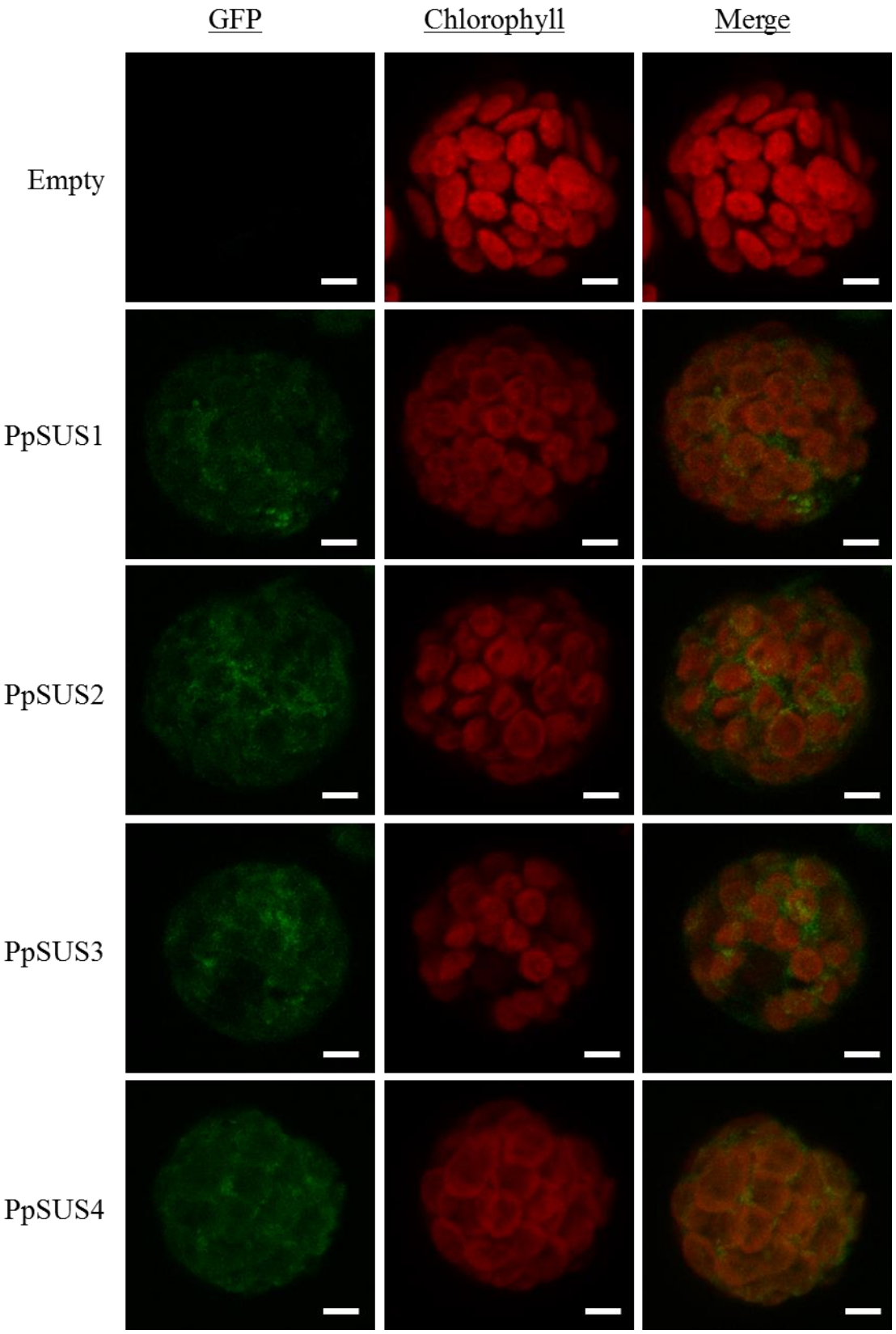

Figure 4.9. Subcellular localization of PpSUS-GFP fusion proteins. Images show GFP fluorescence, chloroplast fluorescence and a merge of the two, reflecting true GFP signals. Protoplasts were transiently transformed with pMP1383::PpSUS-GFP constructs and investigated by confocal microscopy. The negative control represents an untransformed protoplast. Scale bars represent 5 µM.

4.4. Discussion

Sucrose metabolism has been the subject of intense study for several decades due to its centrality in plant growth and development. Much has been revealed about the roles of enzymes involved in its metabolism in angiosperms, however, information is lacking in non-vascular plants. Sucrose synthases (SUS) were of particular interest in this study since it is believed to degrade sucrose before entry into metabolism. It is the only enzyme capable of both synthesizing and degrading sucrose, with the latter direction predominating *in vivo* (Noël and Pontis, 2000). To date, no *SUS* homologs have been cloned and characterized in *P. patens* and in this section I describe our main findings related to the small *SUS* gene family encoded in this bryophytes genome.

4.4.1. Residues necessary for SUS activity are conserved in PpSUS proteins.

Bioinformatic analyses identified four putative *PpSUS* homologs within the *Physcomitrella* genome. The proteins encoded by these contain both glycosyltransferase and sucrose synthase domains. Sequence analyses demonstrated good conservation of amino acid residues necessary for substrate binding within the active sites of both domains (Winter and Huber, 2000, Zheng et al., 2011). Two serine residues subject to phosphorylation in angiosperms (ser-15 and ser-170; Huber and Huber, 1996) are conserved in *P. patens* . In maize the phosphorylation of these residues have been shown to affect SUS localization, with un-phosphorylated proteins localizing to the plasma membrane and phosphorylated proteins localizing to the cytosol (Winter et al., 1997). Furthermore, phosphorylation at ser-170 in maize SUS1 targeted the protein for degradation (Hardin et al., 2003), while phosphorylation at ser-15 affects protein conformation at the amino-terminal which increases catalytic activity (Hardin et al., 2004). These sites have been shown to be phosphorylated by calcium dependent protein kinases (CDPKs, ser-11/15 and ser-170, Fedosejevs et al., 2016; Hardin et al., 2003; Huber et al., 1996) and SNF1 Related Kinases (SnRK1, ser-15, Hardin et al., 2003). *Physcomitrella* encodes a large *CPDK* gene family consisting of 25 genes (Hamel et al., 2014; Mohanta et al., 2015) and homologs of *SnRK1* (Thelander et al., 2009; Wu et al., 2017), but clearly more research needs to be performed to see if they play a similar role in bryophytes. Researchers have proposed that SUS phosphorylation regulates sucrose metabolism in accordance with developmental and environmental cues, switching between synthesis and degradation as needed. The conservation of these serine residues in PpSUS proteins could be two fold. First, phosphorylation could target the protein for degradation to regulate SUS abundance when necessary. It has been

suggested that SUS turnover is required during sink to source transitions of a given tissue (Hardin et al., 2003). Second, phosphorylation could alter SUS localization in concert with the needs of the cell, lending more flexibility to the plant. Overall, protein phosphorylation is implicated in signal transduction, with the addition/removal of phosphate leading to changes in the localization, activation status and catalytic activity of affected proteins.

One cysteine residue (cys-266) known to be thiolated (Hardin et al., 2003; Röhrig et al., 2004) is, however, unconserved in the PpSUS proteins. This amino acid is replaced by aspartic acid in all four proteins. The activity of a soybean SUS has been demonstrated to be affected by ENOD40 thiol peptide binding at cys-264 (equivalent to cys-266 in AtSUS1) and, depending on the peptide that binds, activity was either enhanced (ENOD40A) or unaffected (ENOD40B; Röhrig et al., 2004). Furthermore, peptide binding at in maize SUS1 antagonized phosphorylation at ser-170, with the authors proposing that it enhances protein stability by blocking downstream degradation through phosphorylation (Hardin et al., 2003). A tBLASTn search revealed that *P. patens* encodes *ENOD* genes (data not shown) and so while the cys-266 residue is not conserved in PpSUS proteins, it cannot be ruled out that PpSUS proteins are not thiolated, as it has been demonstrated that ENOD40 peptides bind maize SUS1, where cys-266 is replaced by an alanine residue (Hardin et al., 2003). On the other hand the absence of PpSUS thiolation might imply that the extensive regulation observed in vascular plants may have arisen as a result of the colonization of land by vascular plants. It cannot be ruled out, however, that other residues might be subject to similar post-translational modifications in *P. patens*.

4.4.2. *SUS* genes from land plants differentiate into 5 clades.

Previous phylogenetic analyses have differentiated SUS genes into three major clades: SUSI, SUSII and SUSIII (Stein and Granot, 2019; Wang et al., 2015; Xiao et al., 2014) where sequences within group I are further differentiated into monocot and eudicot subgroups (Jiang et al., 2015). In this study I created a comprehensive maximum likelihood tree using sequences from species capable of photosynthesis, ranging from cyanobacteria to angiosperms. Our phylogenetic analysis included sequences from gymnosperms, bryophytes and green algae alongside vascular plants. As expected the vascular plant sequences divided into three clades and, I observed the formation of three further clades; one for gymnosperms, one for non-vascular plants and one for green algae. This separation of gymnosperm from angiosperm sequences has been reported previously (Stein and Granot, 2019), lending confidence to this analysis. The solitary *SUS* sequence from *S. moellendorffii*, a lycophyte, did not group with

either gymnosperms or bryophytes. Lycophytes represent the oldest lineage of vascular plants, closer to ferns than other land plant groups, explaining this separation from bryophytes and gymnosperms. Within the bryophyte clade, it appears that *PpSUS1* was the original *SUS* gene encoded by *P. patens* as this gene is closely related to the solitary *SUS* gene encoded in *M. polymorpha*'s genome. A duplication event then gave rise to *PpSUS3*, from which further duplications gave rise to *PpSUS2* and *PpSUS4*. It has been reported that *P. patens* has undergone a genome duplication event about. 30 to 60 million years ago with the numbers of genes related to metabolism being expanded (Rensing et al., 2008), which may help explain these duplicates.

4.4.3. Gene expression and protein activity analyses.

Analysis of gene expression can aid in revealing the possible physiological functions of genes. To this end, expression was analyzed in various developmental stages of *P. patens* and over a day/night cycle. The expression of many genes involved in carbohydrate metabolism have been shown to be change over a day/night cycle (reviewed in Kim et al., 2017). To examine this in *PpSUS* genes I analyzed transcript accumulation using RT-qPCR (Figure 4.6). Expression of *PpSUS2, 3* and *4* had similar patterns over all time points, with *PpSUS3-4* showing very little change in expression throughout. *PpSUS2* however, showed a large scale increase at the end of the day. *PpSUS1* showed a distinct expression pattern compared to the other genes, with a steady increase during the day, with expression peaking at the end of the day time point. Expression at this time point saw a 3 fold increase compared to the middle of the day. It would appear, therefore, that only *PpSUS1* and *PpSUS2* are subject to large changes in expression over a day/night cycle where they may be implicated in sucrose metabolism at these time points. To ascertain whether PpSUS proteins were active during these time points, I performed activity analyses using desalted crude protein extracts from the same material. I also assayed neutral INV (nINV) and acid INV (aINV) activities to examine other enzymes involved in sucrose degradation. SUS activity was present in all four time points, and remained largely unchanged, however, I cannot say which of the individual isoforms contributed to the activity. Microarray data demonstrated that *PpSUS1* and *PpSUS2* show highest expression in *P. patens* tissue indicating that they may be the most important isoforms in determining total SUS activity, but this will have to be determined through production and examination of mutants lacking each isoform individually. Unfortunately it proved impossible to ascertain which proteins were catalytically active as all four *Physcomitrella* proteins were quickly degraded

when expressed in *E. coli* (Figure 4.9). Activities for both acid and neutral INV were highest during the middle of the day, decreasing steadily (nINV) or drastically (aINV) nearing the end of the night period (Figure 4.8).

With regards to spatio- and temporal expression, transcript abundance was analyzed using a publically available microarray data set (Ortiz-Ramírez et al., 2016). Here it was revealed that members of the *PpSUS* gene family show some overlapping, yet distinct expression patterns. *PpSUS2* is ubiquitously expressed throughout, with a large scale increase in archegonia, the female gametophyte (Figure 4.5). High sucrose contents have been quantified in the archegonia of the moss *Bryum capillare*, with the authors proposing that these tissues exude sucrose as an attractant for spermatozoids (Kaiser et al., 1985; Ziegler et al., 1988). It is possible that PpSUS2 acts here in the synthesis direction to generate sucrose for this purpose. Furthermore, *PpSUS2* expression increases during sporophyte development, peaking at the S2 stage, after which its expression declines approaching the mature sporophyte. It has been reported that the sporophyte is dependent on the gametophyte for nutrition. Furthermore, the developing sporophyte represents a sink tissue, whereby SUS would be responsible for cleaving sucrose needed to generate not only cellulose for the formation of new cells walls, but also substrates for generating energy. Similar to *PpSUS2*, *PpSUS1* is also highly expressed in archegonia, perhaps supplementing PpSUS2 in this tissue. Furthermore, this genes highest expression can be observed in gametophores at a similar level to that of *PpSUS2*. Interestingly, *PpSUS3* expression is enriched in spores and to a large degree mature sporophytes where it is the most abundant transcript out of all the *PpSUS* genes in these tissues. In spores PpSUS may supply UDP-Glc for the synthesis of callose, which has been shown to be present in spore aperture region (Scheutte et al., 2009). Taken together, the expression and activity analyses support the hypothesis of Volpicella et al. (2016), that different isoforms of the same *SUS* gene family have the same functions, but are active in distinct cell/tissue types, during different developmental stages (*e.g. PpSUS2* in archegonia and *PpSUS3* in mature sporophytes) or under different conditions (*e.g. PpSUS2* activity at the end of the day and *PpSUS1* at the end of the night).

4.4.4. PpSUS proteins localize to the cytosol.

Previous studies on SUS localization have revealed that these proteins have dynamic localization patterns, where they can localize to cytosol (Macdonald and ap Rees, 1983; Nishimura and Beevers, 1979), plasma membrane (Amor et al., 1995), cell walls (Salnikov et

al., 2003), tonoplast (Etxeberria and Gonzalez, 2003), mitochondria (Konishi et al., 2004) and plastids (Núñez et al., 2008). However, SUS is thought to be found predominantly in the cytosol. As sucrose synthesis is thought to exclusively occur in the cytosol, it is unsurprising that all four *Physcomitrella* isoforms localize to that compartment.

4.4.5. The potential role of SUS in sucrose transport

As with many Angiospems, sucrose is the predominant carbon source utilized and translocated in *Physcomitrella* (Allsopp, 1951; Thelander et al., 2009). Hence, it is unsurprising that *Physcomitrella* encodes genes necessary for its metabolism (*e.g. SPS, SPP, SUS, INV*; Thelander et al., 2009) and transport (*e.g.* SUT, SWEET; Reinders et al., 2012; Eom et al., 2015). Even though mosses diverged from the ancestor of seed plants cca. 500 *mya*, they possess specialized cells that conduct water and photosynthate. This offers an opportunity to study SUS function in photosynthate translocation in plants lacking vasculature.

When considering the gametophyte generation of *Physcomitrella*, the different cell types can be considered analogous to source and sink tissues in vascular plants. Chloronema are denser in chloroplasts, serving as source cells that provide photosynthate to caulonema. This could help for both colony expansion (due to caulonemal filaments growing faster) and gametophore development, which develop from caulonemal filaments. Photosynthate translocation between the different cell types have been suggested to be symplasmic (Raven, 2003; Regmi et al., 2017; Reinhart and Thomas, 1981). High densities of plasmodesmata in vascular plants facilitate sucrose movement to the phloem, with phloem loading driven by a sucrose gradient between mesophyll cells and the phloem. In *Physcomitrella*, it could be possible that PpSUS and/or invertase plays a role in establishing and maintaining sink strength in sink cells (caulonema), by cleaving incoming sucrose, thereby maintaining the gradient necessary for the flow of sucrose between tissues. Incoming sucrose cleaved by SUS/INV could be used to produce hexoses for cellular respiration, or as precursors for the synthesis of complex carbohydrates. Furthermore, SUS could also function in the synthesis direction to control sucrose movement, by offsetting the existing gradient, diverting sucrose to other cells. In source cells, they could function to supplement SPS and SPP proteins in the synthesis of sucrose. Of the four *PpSUS* genes, *PpSUS1* and *PpSUS2* are expressed in caulonema, chloronema and gametophores, indicating a possible involvement in these processes.

Within sporophytes, an apoplastic mode of photosynthate transport has been suggested. Specialized transfer cells lie at the gametophyte-sporophyte interface, representing a diffusion barrier for photosynthate (Courtice et al., 1978; Ligrone and Gambardella., 1988; Ligrone et al., 1993). In vascular plants, apoplastic transport involves sucrose translocation *via* phloem, which can be transported into sink cells through sucrose transporters. *Physcomitrella* encodes orthologs of Sucrose Uptake Transporter (SUT; Kühn and Grof, 2010; Reinders et al., 2012) and Sugars Will Eventually be Exported Transporter (SWEET; Eom et al., 2015) which may be involved in this process.

A transcriptomic study examining changes in gene expression during sporophyte development reported that the transition from gametophyte to sporophyte involved alterations in the expression of approximately 25% of genes involved in carbohydrate metabolism (O'Donoghue et al., 2013). Amongst those upregulated were *PpSUT4A* and *PpSUT4C*. Regmi et al., (2017) demonstrated that sucrose is transported to the transfer cells symplasmically and subsequently unloaded by SWEET proteins into the apoplasm. It is then loaded into transfer cells by sucrose/H^+ symporters (SUTs). Some of the imported sucrose gets transported further into the sporophyte *via* seta (the stalk that supports the sporophyte capsule) while the remainder is catabolized by SUS. From this model PpSUS function is two-fold, possibly explaining the high expression levels of *PpSUS2* and *PpSUS3* in the sporophyte and *PpSUS1* and *PpSUS2* in the gametophores that house the seta.

When comparing SUS function in *Physcomitrella* and vascular plants, it would appear as if SUS function has been conserved over the course of evolution. In this context, SUS functions mainly to establish and maintain sink tissues/cells (*e.g.* fruits, tubers etc. in vascular plants; caulonemal cells, sporophyte, archegonia and spores in *Physcomitrella*). This will need to be tested using knock-out lines (discussed further in Chapter 6).

4.5. Conclusion

Sucrose synthases are well studied in higher plants, however, reports on this gene family in non-vascular plants are lacking. This study encompasses the investigation of *SUS* genes in the moss *Physcomitrella patens*. Thanks to the release of its genome, I were able to analyze the *SUS* genes encoded in it using gene expression analyses, protein activity analyses, phylogenetic reconstruction and fusion protein localization. This study contributes to the understanding of SUS function in land plants, especially as it relates to non-vascular plants. Furthermore, our results offer a foundation for future research on unravelling the physiological roles for each of the *PpSUS* genes.

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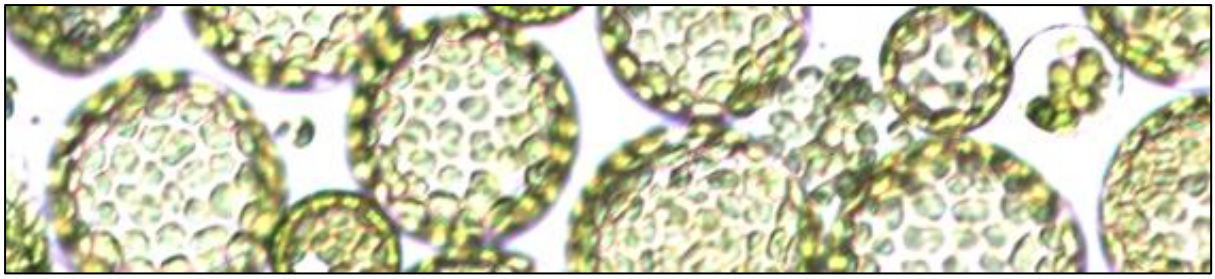

Chapter 5

Characterization of the class II *Trehalose 6-Phosphate Synthase* (*TPS*) gene family in *Physcomitrella patens*

Anna J. Wiese, Ethel E. Phiri, Patrick Van Dijck, Jens Kossmann, David Honys, James R. Lloyd

Author Contributions

The first author (AJW) performed all of the experimental work within the chapter (with the exception of the phylogenetic tree) and wrote the dissertation. With regards to the tree, AJW collected all the sequences and performed initial alignments, while EEP curated the alignment and constructed the tree. PVD provided the plasmid and strains for yeast complementation. JRL conceived of, and supervised the study, while JK and DH provided resources. This chapter is partially written in a style aimed at publication.

5.1. Abstract

Sugars have been shown to possess signaling functions, where they regulate plant growth and development in a manner similar to hormones. Such a function has been demonstrated for trehalose 6-phosphate (Tre6P), an intermediate of trehalose biosynthesis. The proteins involved in the trehalose pathway, namely trehalose 6-phosphate synthase (TPS) and trehalose 6-phosphate phosphatase (TPP), modulate levels of this sugar. TPS polypeptides can be divided into two classes. Class I proteins are catalytically active and are involved in many developmental functions, however, the function of class II proteins remain elusive. Here I report the characterization of class II TPS's in the non-vascular plant *Physcomitrella patens*. *Physcomitrella* contains six *TPS* genes in its genome, two of which encode class I proteins and four class II proteins. Phylogenetic classification differentiated *TPS* sequences from land plants and placed them into 2 clades (I and II), suggesting the existence of one ancestral *TPS* gene. Functional complementation revealed weak TPS catalytic activity for one of the class II TPS proteins. Subcellular localization experiments conducted on three of the class II proteins revealed that they were cytosolic, while yeast two hybrid analyses indicated that these proteins do not form complexes with each other. Finally, expression analyses indicated that class II genes have overlapping expression patterns with regards to developmental stages and expression over a day/night cycle.

5.2. Introduction

Aside from their role in central metabolism, sugars also participate in signaling pathways essential to plant growth and development. One of the sugars implicated in signaling is trehalose 6-phosphate (Tre6P), an intermediate in the trehalose biosynthetic pathway. A role of this sugar in signaling was initially proposed following studies engineering the trehalose biosynthetic pathway into plants in an attempt to improve crop traits (Penna, 2003). Over-expression of bacterial and yeast homologs encoding trehalose biosynthetic genes resulted in phenotypes indicating alterations in carbon metabolism. Further research demonstrated that Tre6P is a key metabolite playing a central role in plant growth and development, acting as a signal for sucrose availability (Ponnu et al., 2011). It has also been found to play roles in flowering (Wahl et al., 2013), seed development (Delorge et al., 2015; Eastmond et al., 2002), starch degradation at night (Figueroa et al., 2016; Martins et al., 2013), abiotic stress tolerance (Foster et al., 2003; Wilson et al., 2007), stomatal conductance (Gómez et al., 2010) and fatty acid synthesis (Zhai et al., 2018).

Trehalose is synthesized by the combined actions of trehalose 6-phosphate synthase (TPS) and trehalose 6-phosphate phosphatase (TPP). Plants in general encode large *TPS* gene families, with the genes subdivided into two classes, with the first generally encoding catalytically active TPSs. No class II protein has been demonstrated to exhibit either TPS or TPP catalytic activities and, therefore, it has been more difficult to determine their precise role in plant growth and development. Expression studies suggest that the genes are not functionally redundant, due to the variability in their expression patterns. Further, the expression of class II encoding genes are regulated by sugars, light, nutrient starvation, diurnal cycles and cytokinins (Contento et al., 2004; Osuna et al., 2007; Price et al., 2004; Ramon et al., 2009; Usadel et al., 2008). In addition to transcriptional control, class II TPS proteins have been shown to be subject to post-translational regulation through phosphorylation and 14-3-3 binding (Glinski and Weckwerth, 2005; Harthill et al., 2006). Class II proteins have also been demonstrated to be members of larger TPS complexes (Zang et al., 2011), with those authors proposing that the complexes are involved in modulating Tre6P levels.

Certain class II TPSs have been shown to be implicated in plants basal defence response against pathogen infection. Silencing the tomato *SlTPS3*, *SlTPS4* and *SlTPS7* genes affected resistance to *B. cinerea* in a negative fashion, while silencing *SlTPS5* led to increased resistance (Zhang et al., 2016). Class II proteins are also implicated in conferring tolerance to abiotic stresses. In *Arabidopsis*, *AtTPS5* is upregulated during heat stress, with *tps5* mutants showing an

impairment in thermotolerance (Suzuki et al., 2008). Similarly, in cotton, *GhTPS11* expression is upregulated in response to heat, PEG, elevated salt and abscisic acid (Vishal et al., 2019). Other roles for class II proteins include its involvement in regulating cell morphology (Chary et al., 2008) and nodule development (Barraza et al., 2016). This lack of overlap in function would suggest that different TPSs have specific or specialized functions.

Bryophytes are excellent models for metabolic studies thanks to the ease with which they are cultured on defined media and the availability of forward and reverse genetic tools. Several species within the basal lineages have been developed over into model plants (*Physcomitrella patens, Funaria hygrometrica, Ceratodon purpureus, Tortula ruralis*) and, as a result, progress has been made in the field of sugar sensing and signaling within these non-vascular plants. For example, studies on carbon and energy metabolism in *P. patens* has demonstrated that moss colonies balance growth between caulonema and chloronema based on energy supply, with the *HXK1*, *Snf1a* and *Snf1b* gene products implicated in monitoring and controlling energy supply (Olsson et al., 2003; Thelander et al., 2007, 2004). Although the two class I proteins have been recently characterized in *P. patens* (Phan et al., 2020), much remains unknown about class II TPSs in both angiosperms and bryophytes. In this study I chose *Physcomitrella* as our model plant to characterize the four class II TPS homologs encoded in its genome. Here I report our main findings as it relates to TPS catalytic activity, protein localization and gene expression.

5.3. Results

5.3.1. *P. patens* contains a small *TPS* multigene family

A TBLASTN search using the *Arabidopsis* AtTPS1 (NP_001322932.1) and AtTPS5 (NP_001329809.1) protein sequences as queries against the Phytozome v3.3 database (phytozome.jgi.doe.gov) revealed 2 putative TPS class I and 4 putative TPS class II orthologs on different chromosomes, designated as PpTPS1-6 (Table 5.1). The two class I protein sequences (PpTPS1 and PpTPS2) had an average percentage identity of 64% towards AtTPS1, while the four class II protein sequences (PpTPS3-6) had an average percentage identity of 65% towards AtTPS5. When a TBLASTN was conducted using AtTPS1 as the query for the class II proteins, an average percentage identity below 36% was revealed (data not shown). This indicates that PpTPS1 and 2 represent class I TPS's and the others class II.

Table 5.1. *Physcomitrella patens TPS* homologs revealed through TBLASTN search. Accessions from Phytozome are included to the right of the gene name I ascribed each locus. The max score describes the quality of the alignment, the query coverage the percentage overlap between the query and the subject, the E value the significance of the score and finally the percentage identity indicates the percentage similarity between the two amino acid sequences.

Query - AtTPS1 (NP_001322932.1)						
	Gene	Phytozome Accession	Score	Coverage	E Value	Identity
Class I	*PpTPS1*	Pp3c5_17730	1101	90%	0	63.60%
	PpTPS2	Pp3c6_16450	1106	89%	0	64.20%

Query - AtTPS5 (NP_001329809.1)						
	Gene	Phytozome Accession	Score	Coverage	E Value	Identity
Class II	*PpTPS3*	Pp3c25_6990	864	82.10%	0	67.20%
	PpTPS4	Pp3c7_15250	870.2	76.70%	0	61.80%
	PpTPS5	Pp3c11_17560	880.6	78.50%	0	65.50%
	PpTPS6	Pp3c16_19150	864.8	79.70%	0	65.00%

Comparison of genomic sequences with coding sequences revealed that *PpTPS1* and *PpTPS2* both contain 19 exons interrupted by 18 introns (Figure 5.1.A). *PpTPS3* and *PpTPS6* contains 4 exons with 3 introns and *PpTPS4* and *PpTPS5* 5 exons and 4 introns (Figure 5.1.A). The coding sequences of class I *PpTPS* genes range from 3096 bp to 3150 bp while class II coding sequences range between 2562 bp to 2676 bp. According to sequences obtained from Phytozome *v3.3* the PpTPS1 and 2 proteins have 1049 and 1031 amino acid residues, while PpTPS3-6 proteins have 856, 891, 891 and 853 residues respectively. A conserved domain

search revealed that all four proteins contain both the glycosyl transferase domain and the trehalose phosphatase domain characteristic of plant TPS proteins (Figure 5.1.B).

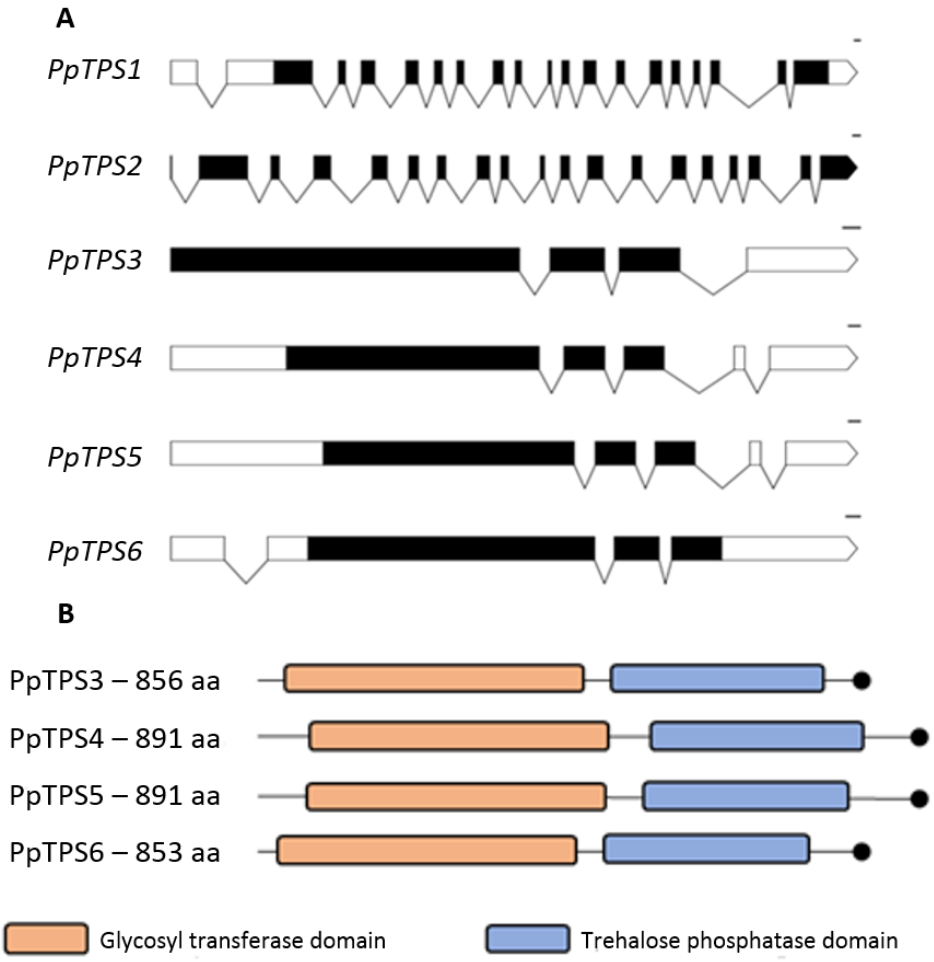

Figure 5.1. Sequence analyses of the class II TPS genes in *P. patens*. (A) Gene structures of all six TPS genes. Scale bars represent 100 bp. White boxes indicate untranslated regions, black boxes exons containing coding sequence and lines introns. (B) Class II proteins possess both glycosyl-transferase and trehalose phosphatase domains.

5.3.2. Phylogenetic analysis of *P. patens TPS* genes

Previous studies have grouped land plant *TPS* genes into class I and class II subfamilies (Han et al., 2016; Lunn, 2007; Yang et al., 2012). We sought to confirm which *PpTPS* genes fall within which class and how *TPS* genes evolved within plants. To this end a phylogenetic tree was constructed using the comprehensive maximum likelihood (ML) method with sequences from green algae (*Chlamydomonas reinhardtii, Volvox carteri, and Ostreococcus lucimarinus*), bryophytes (*Marchantia polymorpha, Physcomitrella patens*), a lycophyte (*Sellaginella moellendorffii*), gymnosperms (*Gingko biloba*) and angiosperms (*Arabidopsis thaliana, Solanum tuberosum, Oryza sativa, Populus trichocarpa*) (Figure 5.2). The *E. coli otsA* sequence was used as an out-group. Coding sequences were collected from Phytozome and NCBI and aligned using MUSCLE (Edgar, 2004), with a maximum likelihood tree constructed using the Tamura-Nei model (Tamura and Nei, 1993) in MEGA X (Kumar et al., 2018).

This analysis differentiated the TPS sequences into two clades encoding either class I or II isoforms. As reported previously (Han et al., 2016; Henry et al., 2014; Yang et al., 2012) there appears to be a further differentiation within both clades, with clade I having 2 subgroups (1A-B) and clade II 5 subgroups (2A-E). Two of the subgroups in class II (2D-E) contain mostly sequences from bryophytes and the lycophyte *S. moellendorffii*, with the exception of 4 sequences from rice in 2D. Within 2E, only *P. patens* sequences can be found. The remaining 3 groups contain sequences from angiosperms and the gymnosperm *G. biloba*. Interestingly, as reported previously (Xu et al., 2017), *S. tuberosum* does not appear to encode any class I genes with all of its sequences fall within the class II clade. With regards to *PpTPS* genes, the class I genes appear to be most similar to genes from *S. moellendorffii*, whereas the class II genes appear to be most similar to TPS II genes from the liverwort *M. polymorpha*. The analysis also revealed that the class II genes within *Physcomitrella* were present as three duplicated gene pairs (*PpTPS5/4, PpTPS6/3* and *PpTPS1/2*).

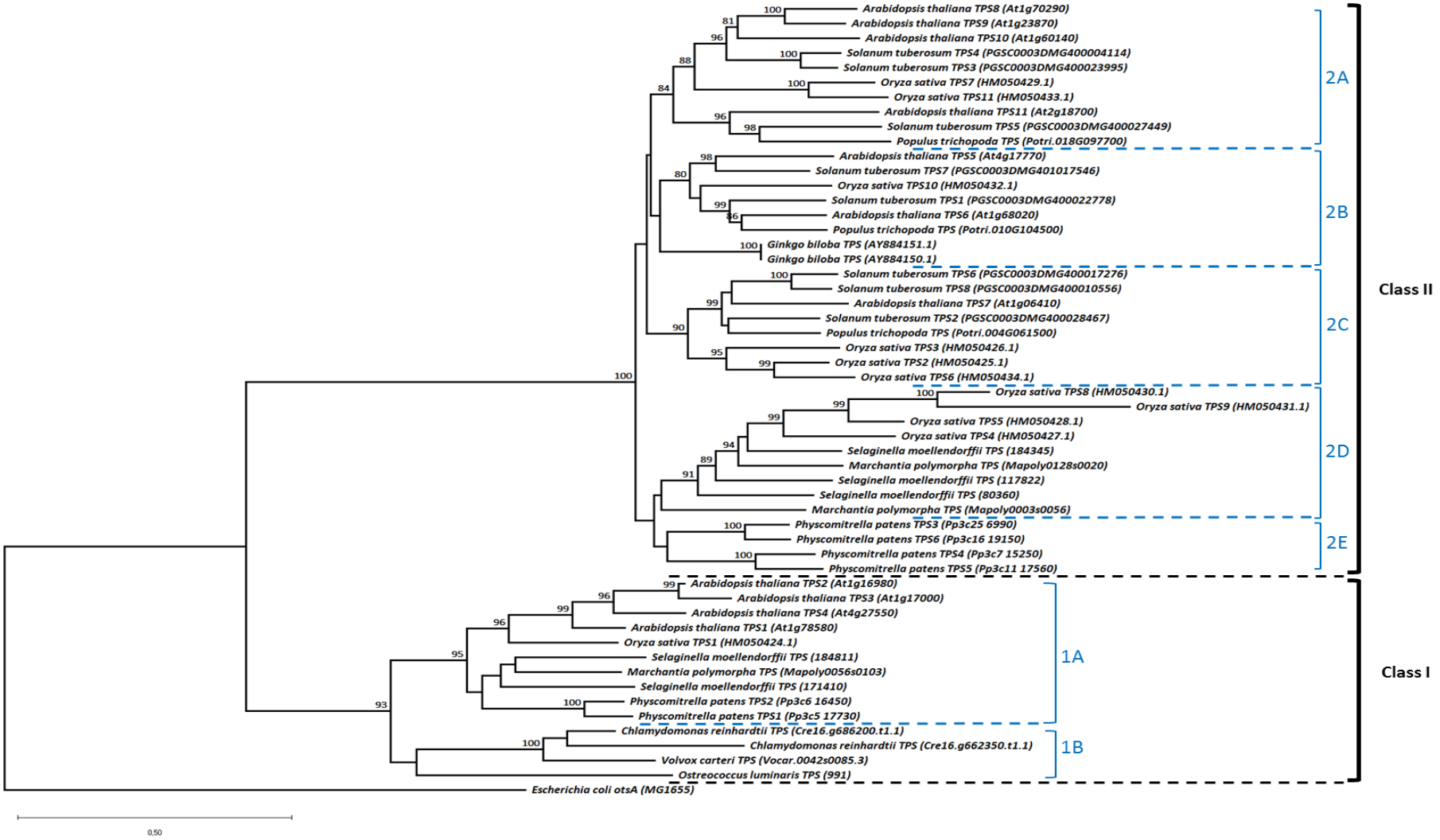

Figure 5.2. Phylogenetic classification of 52 TPS sequences from 12 species. The evolutionary history was inferred using the Maximum Likelihood method and General Time Reversible model (Tamura and Nei., 1993). The tree with the highest log likelihood (-70373.61) is shown. The tree is drawn to scale with branch lengths measured in the number of substitutions per site. Bootstrap values below 75% are not shown. The *E. coli otsA* sequence was used as an outgroup.

5.3.3. Expression analysis of class II genes

Next I examined the expression profiles of the class II genes, alongside the profiles of the two class I genes. Real-time quantitative PCR (qPCR) was used to examine changes in expression over a day/night cycle. Normalization was performed using *PpActin5* expression using middle of the day as a calibrator sample. Results are reported as normalized fold change (Figure 5.3). All four class II *TPS* genes and *PpTPS1* (class I) showed a similar pattern of expression throughout the day/night cycle. Results indicate that transcript accumulation peaks nearing the end of the light period after which expression decreases. A subsequent increase is observed near the end of the night period. Interestingly, *PpTPS2* expression increased steadily peaking at the end of night. A similar trend was observed for the other homologs

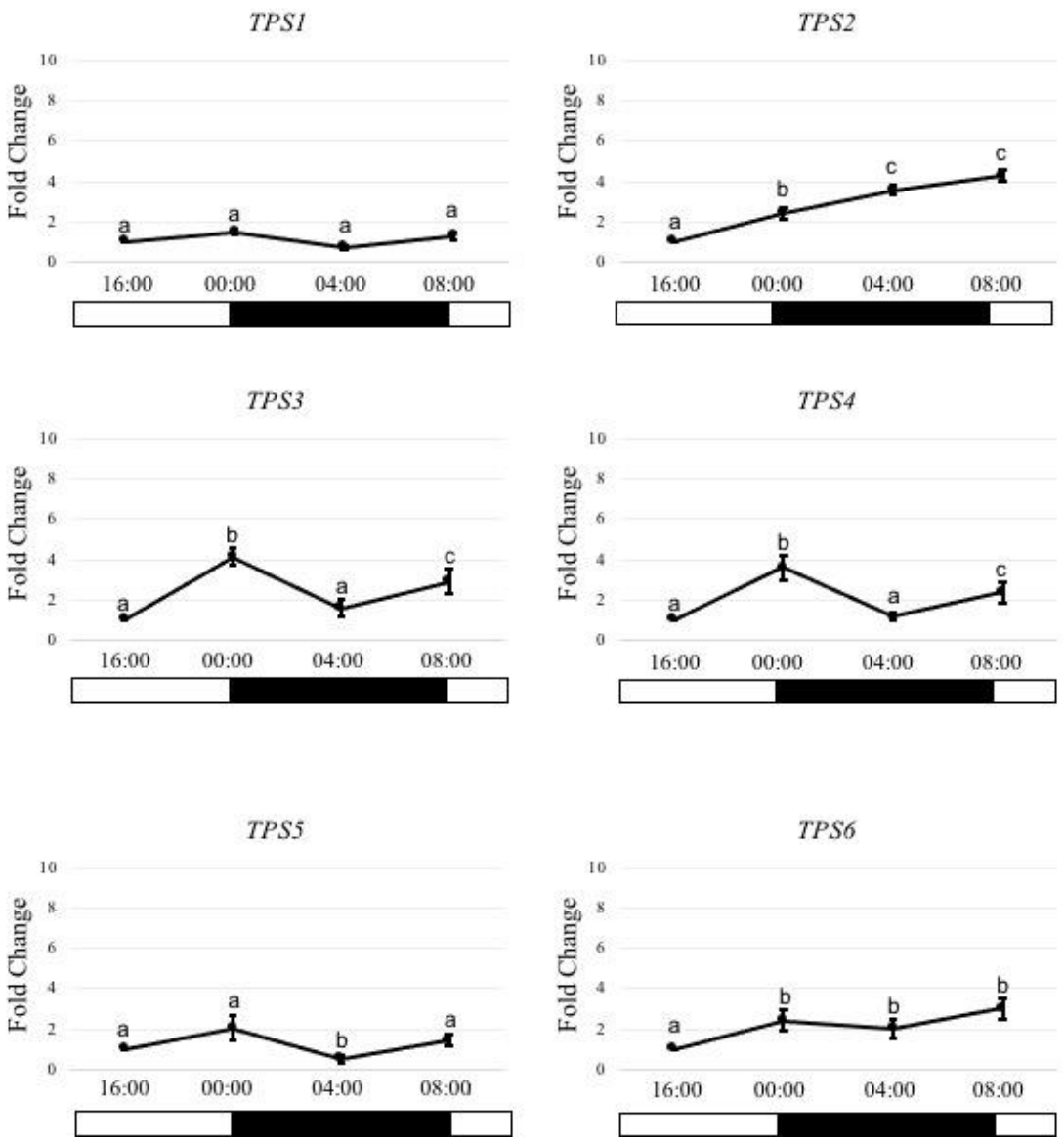

Figure 5.3. qRT-PCR expression of *PpTPS* genes over a day/night cycle. Six-week-old whole moss colonies were collected at the four time points indicated, with one colony representing one biological replicate. Data represents fold change of *PpTPS* relative to the reference gene *Actin* (Pp3c10_17070), using the time point 16:00 as the calibrator sample. Values represent the mean ± standard error of three independent biological replicates calculated from the Ct values using the ΔΔCt method (Livak and Schmittgen, 2001). Error bars represent standard errors, and when not visible, are within the circular symbols. Significant differences are indicated by different letters, as determined by a Bonferroni-Holm *post hoc* test following a one way ANOVA. A value of 1 represents no expression deficiency. Black boxes indicate the dark periods and white boxes, the light periods.

in silico expression analyses of *P. patens* developmental stages and tissue types indicated that the class I genes are the most highly expressed. Expression for *PpTPS1* was especially enriched in gametophores, archegonia and during sporophyte development. The same was observed for *PpTPS2*, albeit to a lesser extent. As for the class II genes, *PpTPS4* showed ubiquitous and high levels of expression throughout development (Figure 5.4). Gametophore enriched expression was observed for *PpTPS3*, as well as an increase in expression during sporophyte development. Similarly, *PpTPS5* had high expression in gametophores and an increase in expression during sporophyte development. Finally, *PpTPS6* had highest expression in gametophores, rhizoids, spores and certain stages of sporophyte development.

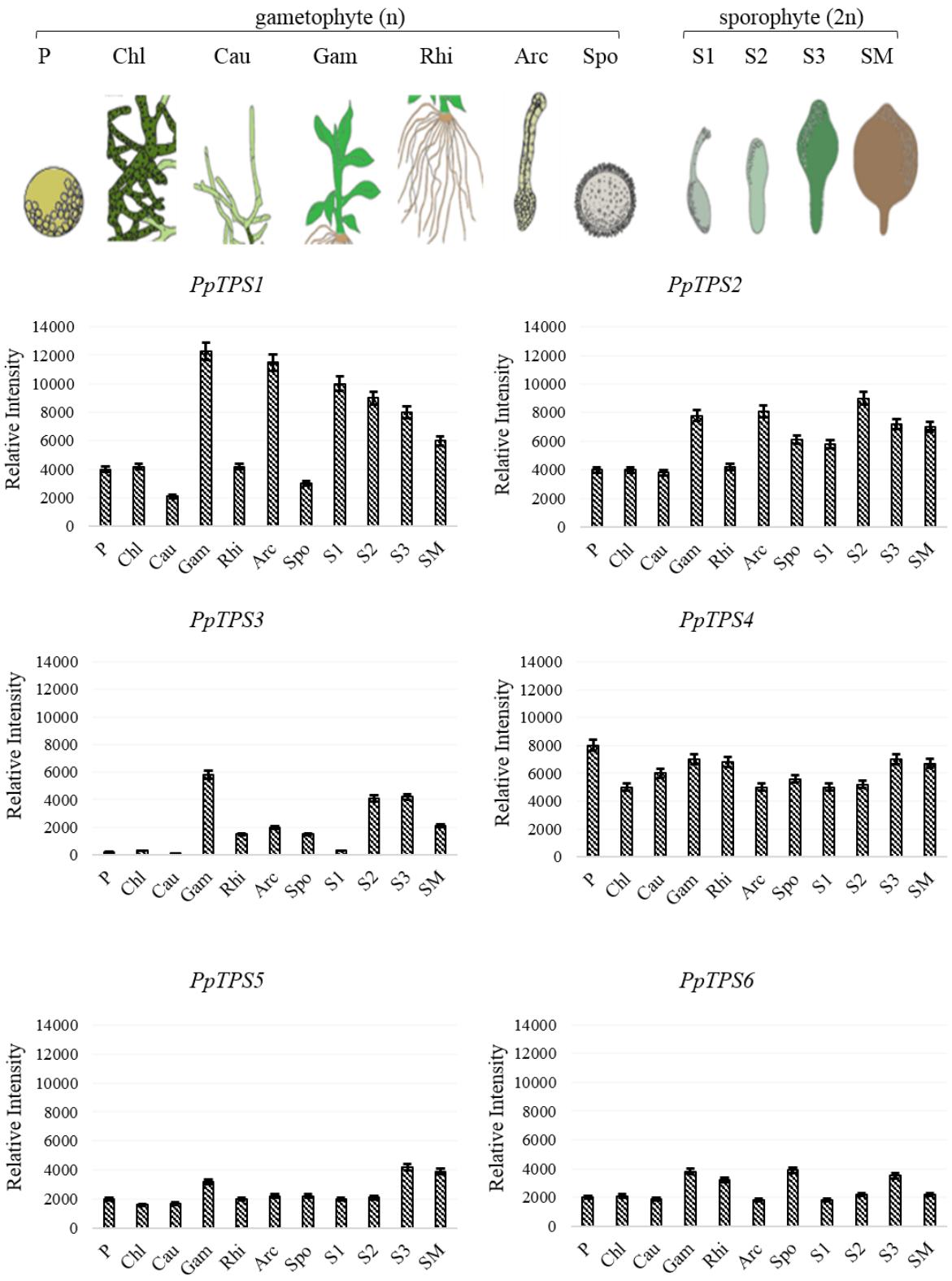

Figure 5.4. *in silico* expression analyses of *PpTPS* genes in different developmental tissues. Data were obtained from eFP browser which is a repository of publicly available transcriptomic data sets. Data are from Ortiz-Ramírez et al. (2016). Acronyms include: P = protoplast; Chl = chloronema; Cau = Caulonema; Gam = gametophyte; Rhi = rhizoids; Arc = archegonia; Spo = spore; S1 – S3 = different stages of sporophyte development; SM = mature sporophyte.

Further *in silico* expression analysis was conducted to reveal how transcript accumulation changes in response to stress (heat and dehydration) and hormone (auxin and strigolactone) treatments (Figure 5.5). Previous studies have demonstrated that class II proteins show unique expression profiles in response to these treatments (Mu et al., 2016; Ramon et al., 2009). Strigolactone treatment led to an upregulation in the expression of *PpTPS3* and *PpTPS5*, while leading to a downregulation in the expression of *PpTPS6*. Expression of the remaining genes appeared to be unaffected by the treatment. Auxin treatment lead to a slight downregulation in the expression of *PpTPS3* and *PpTPS6* only. Gametophores subjected to dehydration and rehydration showed a similar profile in the expression of all 6 *PpTPS* genes. The expression of each gene was downregulated (severely (*e.g. PpTPS6*) or mildly (*e.g. PpTPS5* and *PpTPS3*)). The expression of *PpTPS2* and *PpTPS4* were very lowly expressed in gametophores in the first place. Heat stress led to an upregulation in the expression of *PpTPS5* (6-fold increase), while it led to a downregulation in the expression of *PpTPS6* and *PpTPS3*.

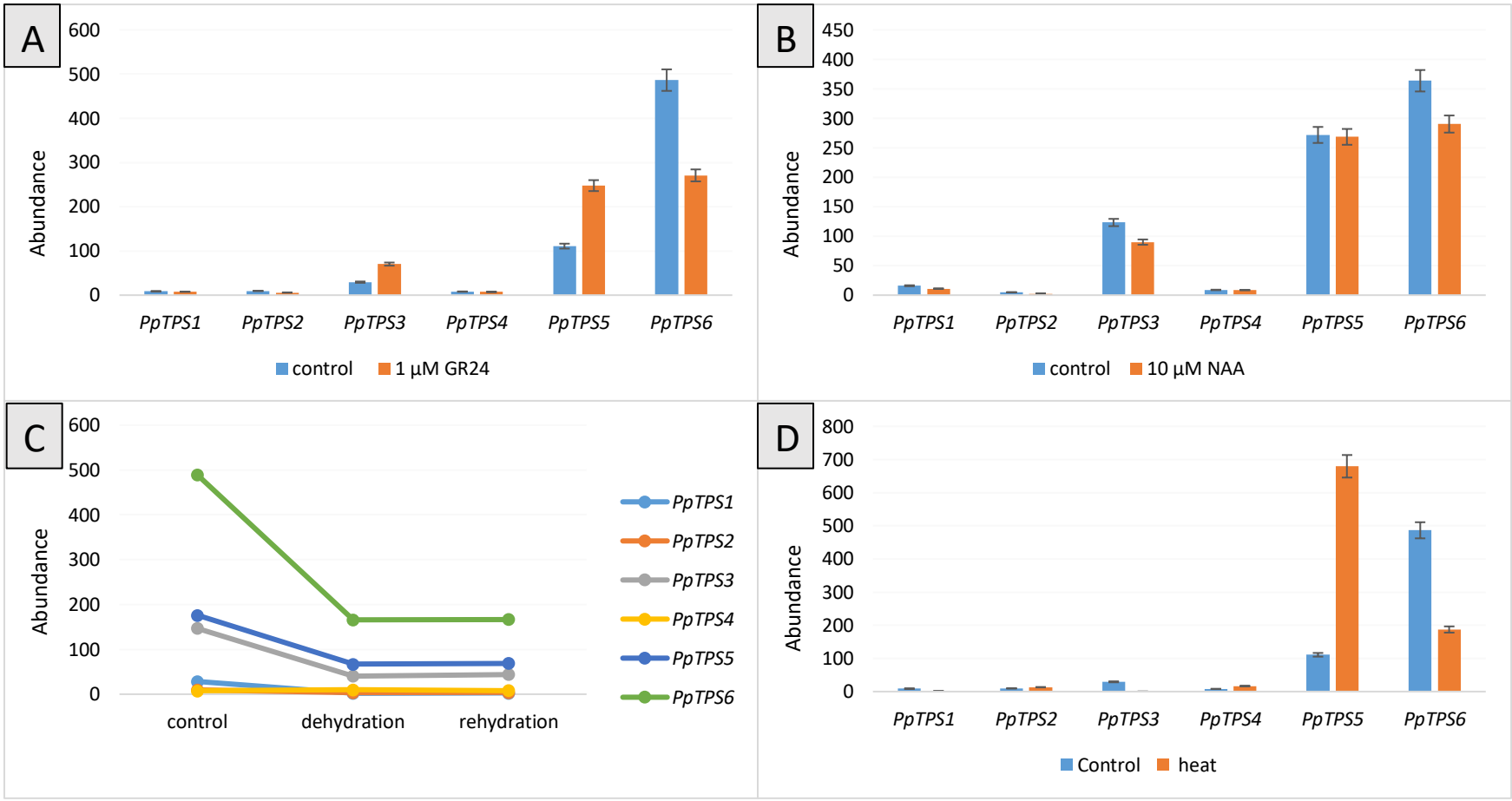

Figure 5.5. In silico expression analyses of *PpTPS* genes in response to hormone and stress treatments. Data were obtained from Phytozome's PhytoMine repository which contained publicly available transcriptomic data sets. Data are from Perroud et al. (2018). (A) Response to strigolactone treatment. Protonemal tissue cultured on BCD plates supplemented with acetone (control) or 1µM GR24 dissolved in acetone (strigolactone). (B) Response to auxin treatment. Gametophores cultured above liquid Knop media supplemented with 10 µM IAA (auxin) or without (control). (C) Response to de- and re-hydration. Five-week-old gametophores cultured on BCD media were moved to empty petri dishes for 180 h (dehydrated) and rehydrated on sterile water for 5 min following which they were moved to BCD plates for 2h prior to sampling. (D) Response to heat stress. Protonema cultured on BCD media were subjected to heat stress treatments (5 h at 22ºC, 1 h at 37ºC) for 5 days and sampled.

5.3.4. Sequence analyses reveal a good conservation of active sites necessary for activity.

The glycosyltransferase domain utilizes UDP-glucose (UDP-Glc) as a donor molecule and glucose 6-phosphate (Glc6P) as an acceptor for the synthesis of Tre6P. The *E. coli* TPS (otsA) has been well characterized, with known active sites that bind the substrates (Gibson et al., 2002; Withers et al., 2002). Alignment of the TPS domains of the four class II TPS proteins with otsA shows surprisingly good conservation of acceptor sites (± 86%; Figure 5.6) where 6 of the 7 amino acids (AA) known to be involved in catalysis are conserved. The exception is at the Arg-9 site in otsA (R9A) where arginine is replaced with alanine in all four *P. patens* proteins. With regards to donor sites, four show complete conservation (L154, K268, E370, D362), four sites conservative replacements (i.e. replaced by an amino acid (aa) with similar biochemical properties; R263D, F340V, Q189H, M364L) and one radical replacement (i.e. replaced by an aa with different biochemical properties; G22D for PpTPS4 and PpTPS5).

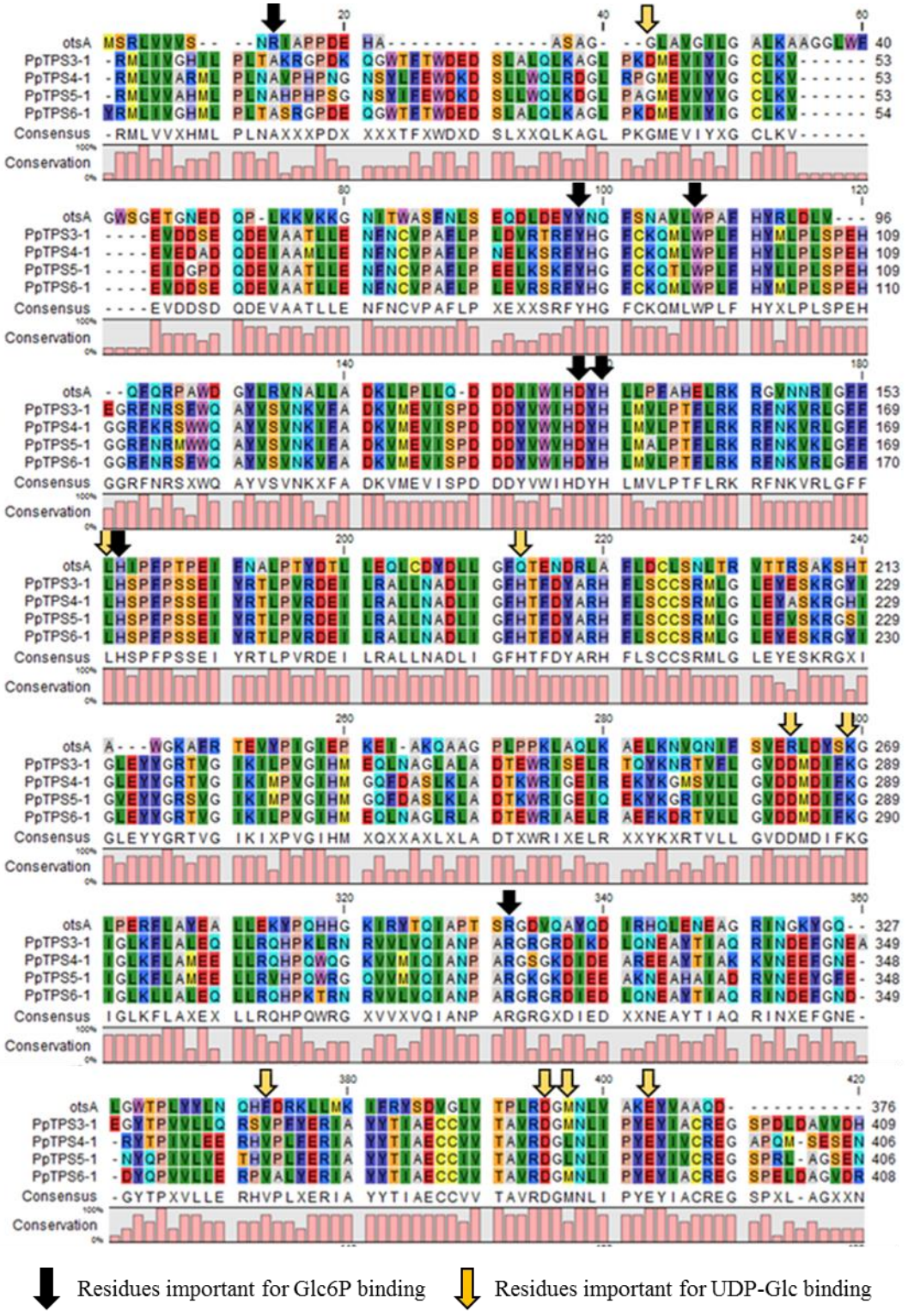

Figure 5.6. Multiple sequence alignment of *Physcomitrella* class II TPS glycosyltransferase domains and *E. coli* otsA. Protein sequences were aligned in CLC workbench using the progressive alignment algorithm, with the level of conservation indicated in the bar chart below the alignment.

Alignment of the TPP domains present in the four class II TPS proteins with *E. coli* TPP (otsB) revealed that ± 68% of the active sites (Avonce et al., 2006; Vandesteene et al., 2010) are conserved, while 2 out of the 7 non-conserved sites show conserved replacement (V195I, T237S), and the other five radical replacements (L21Y, E27P, P32N/H, L152W, K193A) (Figure 5.7).

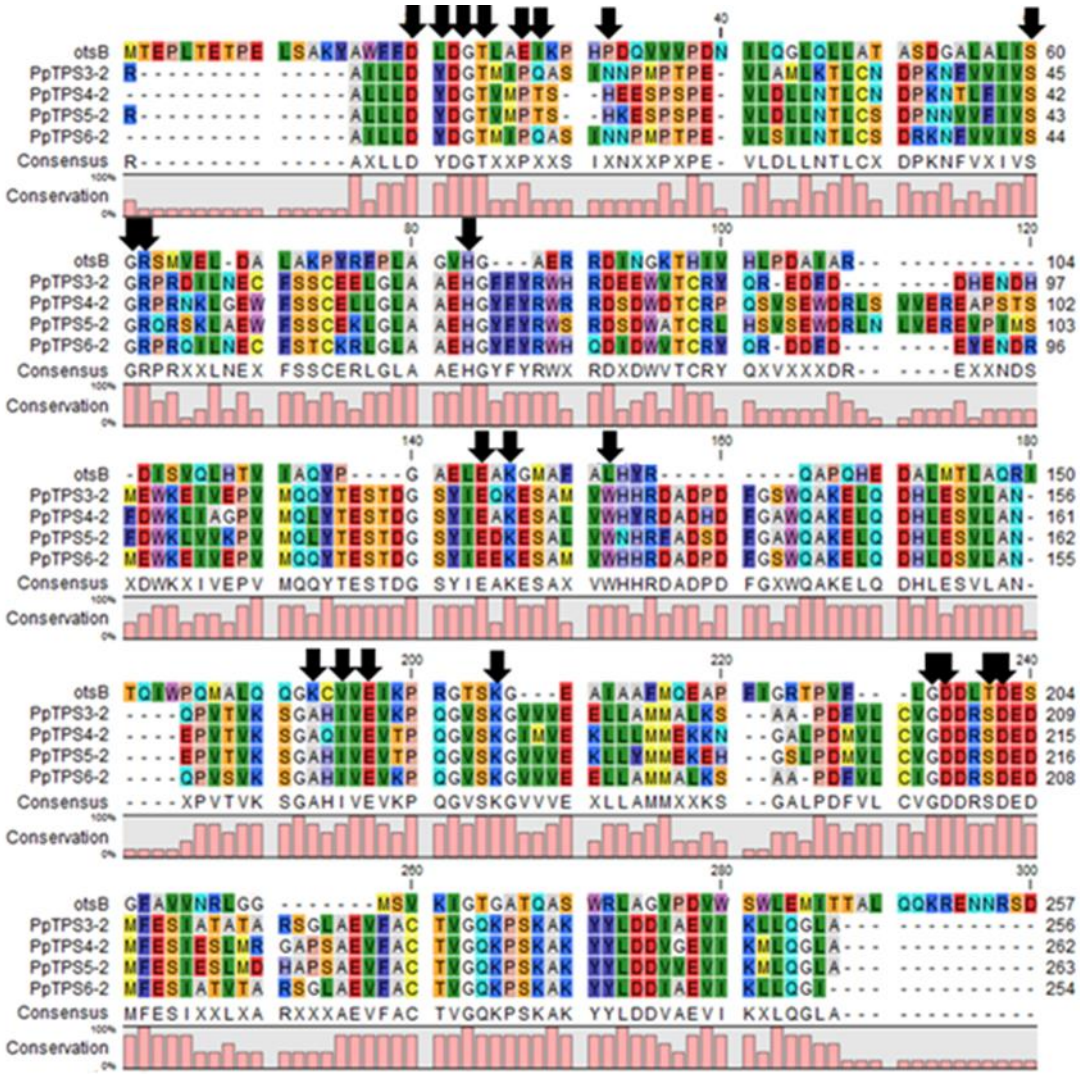

Figure 5.7. Multiple sequence alignment of *Physcomitrella* class II TPS trehalose phosphatase domains and *E. coli* otsB. Protein sequences were aligned in CLC workbench using the progressive alignment algorithm, with the level of conservation indicated in the bar chart below the alignment. Residues important for Tre6P binding are 1indicated in black arrows.

5.3.5. Yeast complementation reveals TPS activity in a single class II protein.

Based on the high overall sequence similarity observed between otsA and the class II TPS proteins, I examined whether they possess catalytic activity using yeast complementation. The *tps1Δ* strain is unable to grow on minimal media plates containing glucose due to a depletion of ATP (Teusink et al., 1998; Chapter 2, section 2.6.3). This can be overcome by growing *tps1Δ* mutants on galactose as utilization of this sugar is unregulated by hexokinase (HXK), the enzyme that controls entry of glucose into glycolysis. Similar to the *tps1Δ* mutant, the *tps1Δtps2Δ* double mutant is unable to grow on minimal media with glucose as a sole carbon source but can do so when galactose is supplied. The *tps2Δ* strain is thermosensitive due to an excessive accumulation of Tre6P at temperatures above 38.5°C, making these temperatures the selector for catalytically active TPP proteins. To complement the mutants, CDSs lacking stop codons were cloned into the pYX212 expression vector, containing the constitutive HXT7 promoter, in frame with a human influenza hemagglutinin (HA) tag. The vectors, including positive controls (pYX212::ScTPS1-HA and pYX212::ScTPS2-HA) were introduced into the different strains and growth on selection plates scored. As expected, all of the transformed strains grew equally well on galactose while none of the class II TPSs were able to restore growth on glucose plates in the *tps1Δ* background (Figure 5.8.A). Surprisingly however, PpTPS5 was able to restore growth in the *tps1Δtps2Δ* background as effectively as ScTPS1 (Figure 5.8.B). No PpTPS protein led to growth of a *tps2Δ* strain (Figures 5.8.C). These results indicated that class II TPSs were unable to complement the heat stress sensitive phenotype when grown at 38.5°C, demonstrating that they do not possess TPP activity. Using immunoblotting, heterologous expression levels of the HA-tagged proteins in the mutant backgrounds were compared (Figure 5.8.D). Full-length proteins were shown to be expressed in all backgrounds.

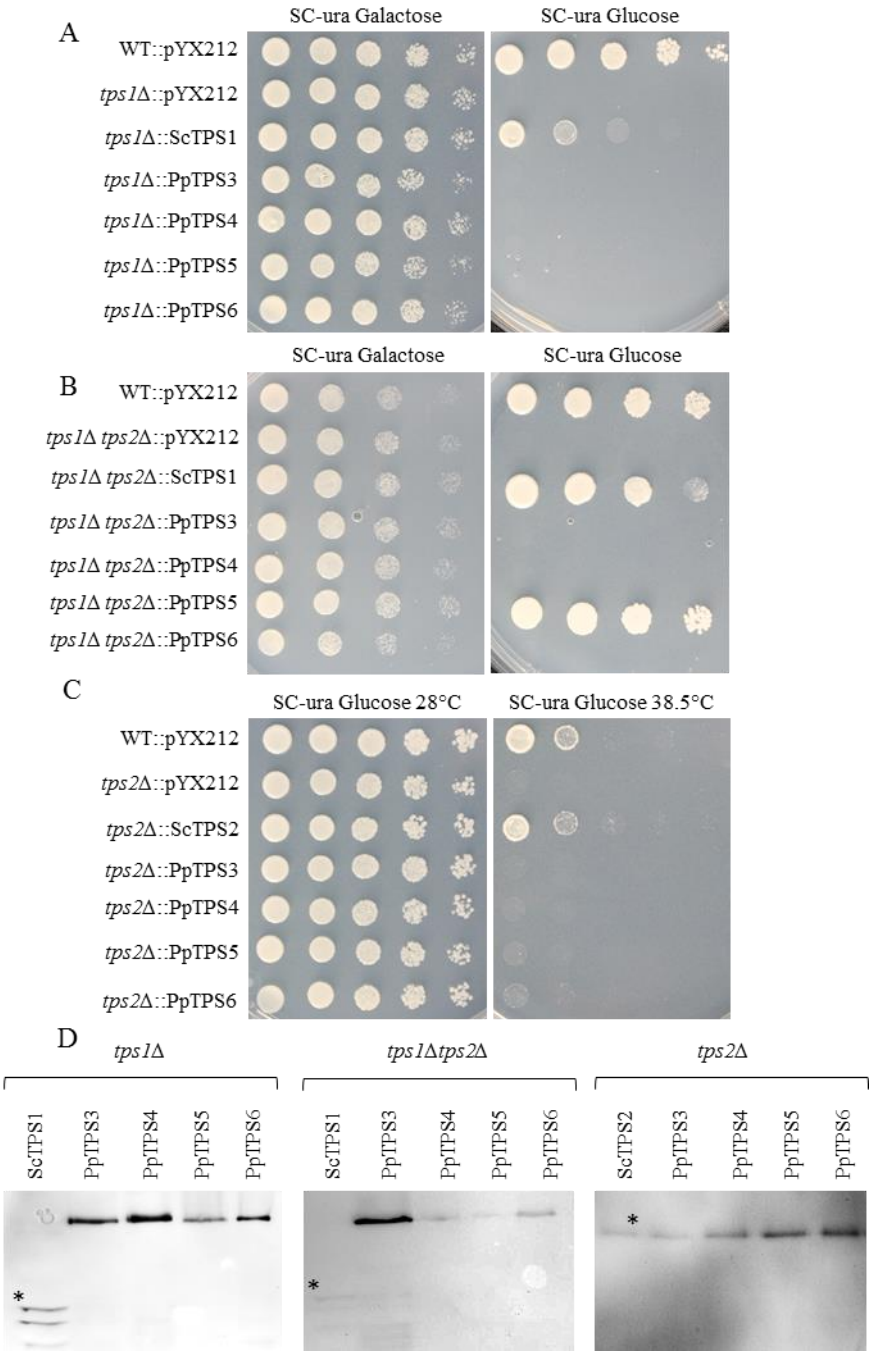

Figure 5.8. Functional complementation of yeast deletion strains using *P. patens TPS* Class II homologs. (A) Yeast strain defective in TPS activity (*tps1*Δ) complemented with control (*ScTPS1*) and *PpTPS* coding sequences. (B) Yeast strain defective in both TPS and TPP activities (*tps1*Δ*tps2*Δ) complemented with control (*ScTPS1*) and *PpTPS* coding sequences. (C) Yeast strain defective in TPP activity (*tps2*Δ) complemented with control (*ScTPS2*) and *PpTPS* coding sequences. As negative controls WT and mutant strains were transformed with pYX212. (D) Immunoblot analysis confirming expression of TPS proteins in the deletion strains. Asterisks indicate yeast ScTPS1-HA (56.16 kDa) and ScTPS2-HA (102.99 kDa) proteins. The four PpTPS proteins are of similar size (PpTPS3-HA = 97.12 kDa; PpTPS4-HA = 101.4 kDa; PpTPS5-HA = 100.75 kDa; PpTPS6-HA = 96.76 kDa).

5.3.6. Class II TPS proteins localize to the cytosol.

To examine the subcellular localization of the four class II PpTPS proteins, GFP fusions were cloned under the control of the constitutive 35S promoter in pMP1383 vectors for transient expression in *Physcomitrella* protoplasts. I was unable to clone PCR amplicons of *PpTPS4* into Gateway entry vectors or ligate them directly into the GFP expression vector. The inability to clone some genes into such vectors has been reported previously (Kirioukhova et al., 2011; Nilsson et al., 2011; Reumann et al., 2009; Xiao et al., 2017), although the reasons for this are unknown. A negative control included untransformed protoplasts. After a 24 h dark incubation period, GFP emissions were detected using a confocal microscope and images captured. Chlorophyll auto fluorescence revealed the chloroplasts, while GFP emissions appeared to come from the spaces between them, indicating that the fusion proteins are localized to the cytosol (Figure 5.9).

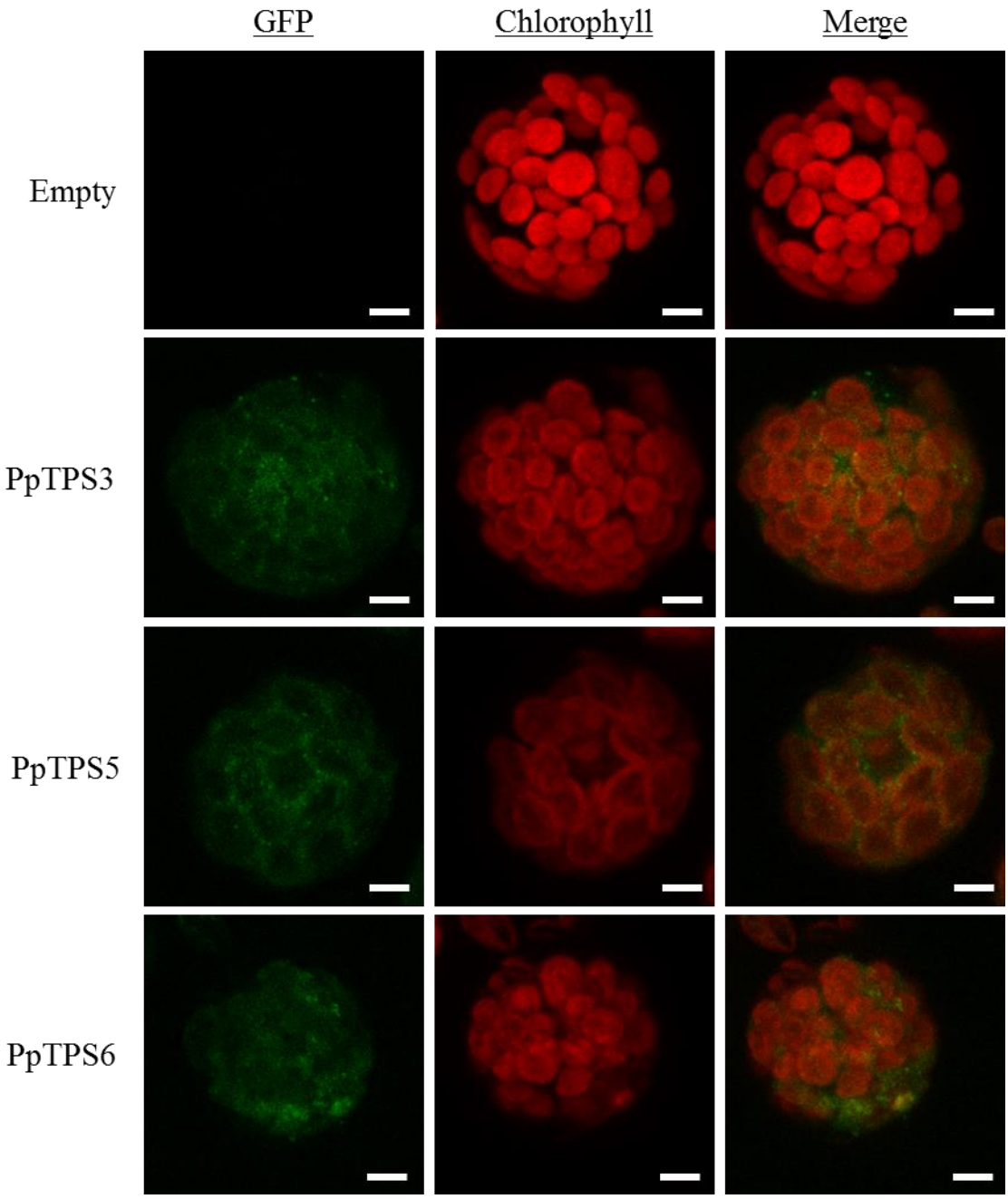

Figure 5.9. Subcellular localization of PpTPS-GFP fusion proteins. Protoplasts were transiently transformed with pMP1383::PpTPS-GFP and investigated by confocal microscopy. Images show GFP fluorescence, chloroplast auto fluorescence and a merge of the two.

5.3.7. *Physcomitrella* TPS proteins do not appear to interact with each other.

In yeast the ScTPS3 and ScTSL1 proteins form part of the trehalose synthesis complex (Bell et al., 1998; Reinders et al., 1997). These proteins lack TPS and TPP activity and are proposed to be regulatory subunits of the complex. Since class II TPS proteins from most plants have not shown any TPS or TPP activity, researchers have proposed that they fulfil regulatory functions potentially through forming protein complexes. To examine if PpTPS proteins form complexes with other PpTPS proteins, I carried out yeast two-hybrid (Y2H) analyses. For the Y2H analysis, full length CDS of both class I and II genes were cloned into pDEST32 and pDEST22 constructs in frame with an N-terminal DNA binding domain (DBD) and an N-terminal activation domain (AD) of the GAL4 transcription factor respectively. As with the GFP fusion vectors above I was unable to clone PpTPS4 in appropriate bait or prey vectors. All other combinations (homo- and hetero-dimerization) were co-transformed into the MaV203 yeast strain and interactions tested on four phenotyping plates. These included -leu/-trp/-his/+75 mM 3AT, -leu/-trp/-ura and -leu/-trp/+5FOA. Overnight cultures were diluted to an OD_{600} of 0.25 and spotted onto plates with incubation at 30^0C for 3 days, after which growth was analysed. None of the combinations introduced into MaV203 indicated that the TPS proteins interacted with each other or themselves (Figure 5.10). Positive selection plates (-ura and -his + 75 mM 3AT) showed no growth while negative selection plates (+ 5FOA) showed no growth repression (Figure 5.10).

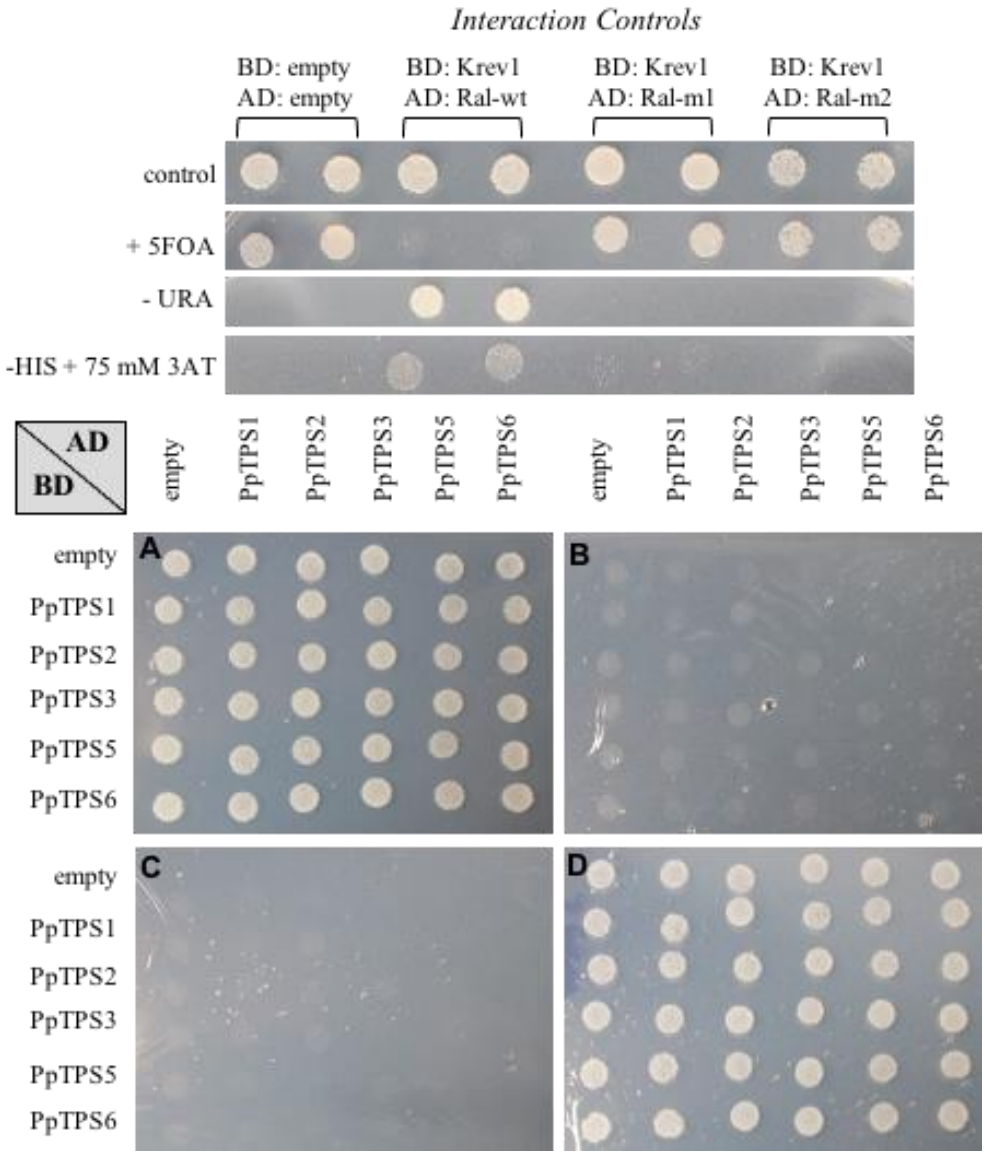

Figure 5.10. Yeast two hybrid analysis of *Physcomitrella* TPS proteins. The MaV203 strain was co-transformed with bait and prey plasmids and transformants spotted onto the following selection plates: (A) SC-leu/-trp (control), (B) SC-leu/-trp/-his + 75mM 3AT, (C) SC-leu/-trp/-ura (D) and SC-leu/-trp/+ 0.1% 5FOA. Growth was scored after 3 days and compared to interaction controls supplied with the kit.

5.4. Discussion

Trehalose 6-phosphate (Tre6P) is crucial to plant growth and development, where it acts as a signal for sucrose availability (Eastmond et al., 2002). Altering Tre6P levels through transgenesis yields plants with pleiotropic phenotypes, suggesting its involvement in various aspects of development (Romero et al., 1997). The enzymes responsible for Tre6P synthesis are TPSs and plants encode large *TPS* gene families differentiated into two classes, with class I proteins being relatively well characterized (Eastmond et al., 2002; Vandesteene et al., 2010). In this chapter I report the examination of four class II homologs in the moss *P. patens*.

5.4.1. *P. patens* encodes a small *TPS* gene family, with members differentiating into 2 clades.

Interrogation of the *P. patens* genome demonstrates that it encodes a total of 6 *TPS* genes, divided into two classes, with class I containing *PpTPS1-2* and class II *PpTPS3-6*. The two class I genes had a percentage identity to the class I *AtTPS1* above 60%, whereas the remaining class II genes had a percentage identity below 36%, however, their percentage identity towards the class II *AtTPS5* was on average 65% (Table 5.1). This gave a preliminary indication of the separation of the genes between the two classes. Gene structures revealed that the two classes also differed in terms of intron density (Figure 5.1). The class I genes have 18 (*PpTPS1*) and 19 (*PpTPS2*) exons while class II genes contain 4 (*PpTPS3/6*) or 5 exons (*PpTPS4/5*). The number of introns and exons further highlight the two duplicated gene pairs within the class II genes. Studies of class I *TPS* genes in *Arabidopsis*, rice and *Populus* have shown that they are more intron rich than class II genes (Yang et al., 2012). Introns have been shown to be beneficial by increasing protein diversity *via* alternative splicing (Gorlova et al., 2014), regulating gene expression (Yang et al., 2013) and by playing a role in mRNA export (Maniatis and Reed, 2002). Genes encoding proteins involved in metabolic pathways are more intron rich than other gene types in *Arabidopsis*, with the authors suggesting the main reason for this being increased protein versatility (Mukherjee et al., 2018). *Physcomitrella* most likely retained the introns in the class I genes for this purpose.

Phylogenetic classification of *TPS* sequences further confirmed the separation between the classes (Figure 5.2). Angiosperm sequences differentiated into two clades, as reported previously (Han et al., 2016; Yang et al., 2012). These were further differentiated into 2 sub-clades within class I sequences and 5 class II sub-clades, indicating that the genes within each sub-clade and main clade are of similar origin and, by extension, the existence of one ultimate

ancestral *TPS* gene for land plants. Currently, there are not enough genomes sequenced form basal land plants to confirm this and this hypothesis will need be examined once these are available. The size of the *TPS* gene family is also expanded in *P. patens* compared with another bryophyte, *M. polymorpha*, which only contains 3 *TPS* genes in total. The six *TPS* genes identified in *P. patens* appear to consist of three gene pairs (*PpTPS1/2*, *PpTPS3/6* and *PpTPS4/5*). This is most likely the result of the genome duplication event that *P. patens* underwent approximately 60 *mya*, where genes involved in metabolism were amplified (Rensing et al., 2008). The reason for this is unclear, but the presence of gene pairs could help impart plasticity to metabolic processes in the event of a mutation in one of the genes eliminating its activity. Furthermore, gene duplication can also lead to functional divergence, with several evolutionary fates (non-functionalisation, neo-functionalisation and sub-functionalisation) for the duplicated genes (Yang et al., 2012).

5.4.2. Overlapping and distinct expression patterns among the class II *PpTPS* genes.

In vascular plants, class II *TPS* genes are extensively regulated at the transcriptional level, with different expression patterns with regards to tissue-specific expression and metabolic status (Ramon et al., 2009; Wang et al., 2019; Yang et al., 2012). Our expression analyses revealed that *P. patens* class II *TPS* genes demonstrate similar expression patterns, albeit with some differences in terms of the amount of RNA accumulation, over a day/night cycle and in different stages of development (Figures 5.3 and 5.4). Over a day/night cycle, all four class II genes showed very similar profiles to the class I *PpTPS1* gene. The level of expression, however, differed slightly with *PpTPS3* and *PpTPS4* showing larger fold changes at the end of the day. The only gene with a unique expression profile over a day/night cycle was *PpTPS2*, the second class I gene, which accumulated more mRNA at the end of the night period which could indicate a requirement for *PpTPS2* specifically at this time point. Based on studies in *Arabidopsis*, it has been proposed that Tre6P interacts with the clock to set the rate of starch mobilization at night, to ensure that reserves are not exhausted at the end of the night period (Anjos et al., 2018; Martins et al., 2013). Catalytic activity for both class I PpTPS proteins has been demonstrated (Phan et al., 2020). It is, therefore, possible that PpTPS2 participates in this process by regulating Tre6P levels at night. The expression of *PpTPS1* did not change considerably over a day/night cycle, however, it is still expressed at the end of the day. During this time point, sucrose levels exceed sink demand, it accumulates in source leaves, leading to a rise in Tre6P, which activates phosphoenolpyruvate carboxylase (PEPC) and nitrate reductase

(NR). This in turn leads to photoassimilates being partitioned away from sucrose synthesis and towards organic acid and amino acid synthesis (needed to alleviate sink limitation), subsequently lowering the amount of sucrose (Figueroa et al., 2016). Starch and soluble sugars have been shown to accumulate over a day/night period in *Physcomitrella* in a similar pattern to *Arabidopsis* (Mdodana et al., 2019). Therefore, PpTPS1 might be implicated in coordinating carbon and nitrogen metabolism and source-sink relations. The class II genes had a similar expression profile, with transcript accumulation not varying a lot over the different time points. Like *PpTPS1*, they are still expressed at these time points, indicating that they also participate in these processes, however, in which capacity is uncertain.

With regards to tissue specific expression the class I genes were the most highly expressed in gametophores, archegonia and during sporophyte development. *PpTPS4* was the most highly expressed of the four class II genes, showing ubiquitous and high levels of expression in all tissues, indicating that its gene product is necessary throughout development. The only class II gene that demonstrated expression in specific tissue types was *PpTPS3*, which showed enriched expression in gametophores and sporophyte development stages S2-MS, suggesting it has a specialised role in these tissues.

In vascular plants, class II *TPS* genes show differential expression with regards to cytokinin treatment and various biotic and abiotic stresses (Jin et al., 2016; Ramon et al., 2009; Yang et al., 2012). To reveal whether this holds true for class II *PpTPS* genes, I conducted *in silico* expression analyses using RNA-seq data generated by Perroud et al. (2018; Figure 5.5). Here I show that three of the class II genes show differential expression with regards to hormone and stress treatments. Strigolactone treatment led to the upregulation of *PpTPS3* and *PpTPS5*, while it led to the downregulation of *PpTPS6*. It has been demonstrated that *P. patens* produces strigolactones which control filament extension and interactions with neighbouring colonies (Hoffmann et al., 2014; Proust et al., 2011). An upregulation of *PpTPS3* and *PpTPS5* indicate that they may be implicated in these processes. Auxin treatment did not elicit a large response, showing only slight downregulation in the expression of *PpTPS3* and *PpTPS6*, .similar to class II genes in *Arabidopsis*, class II (Ramon et al., 2009). With regards to stress treatments, *PpTPS* expression was downregulated for all genes upon dehydration, with very little recovery in expression levels following rehydration. It would appear, therefore that *P. patens* does not utilize this pathway for the acquisition of drought tolerance. This is interesting, as the expression of some *TPS* genes in other plant species show an upregulation during drought stress (Nepomuceno et al., 2002; Wang et al., 2019), with authors proposing these TPS proteins to be

implicated in drought tolerance. Finally, heat stress elicited a large change in expression of *PpTPS5*, which was upregulated 6-fold. The expression of *PpTPS6* and *PpTPS3* on the other hand was downregulated, but to a lesser degree with regards to fold change. It would appear, therefore, that *PpTPS5* may play a specific role during heat stress in *P. patens*.

As mentioned above (section 5.4.1), gene duplication can also lead to functional diversification. These include non-functionalisation (one gene becomes a pseudogene, while the other retains its original function), neo-functionalisation (one gene acquires new functions, while the other retains its original function) and, sub-functionalisation (each gene retains some of the functionality of the original gene). For the *PpTPS6/3* gene pair, both had similar expression profiles in all time points, tissues, hormone and stress treatments (*e.g.* downregulated by auxin and heat). This suggests that *PpTPS3* underwent sub-functionalisation, retaining some of the functions of the parent gene. For the *PpTPS5/4* gene pair, differential expression patterns were observed in different tissues and in response to strigolactone treatment and heat stress. This suggests that *PpTPS4* may have acquired a new function *via* neo-functionalisation from *PpTPS5*.

5.4.3. PpTPS5 demonstrates TPS catalytic activity.

All of the class II proteins contain both a glycosyltransferase and a trehalose phosphatase domain, characteristic of plant TPS proteins. Sequence alignments of both domains alongside the well characterized *E. coli* TPS (otsA) or TPP (otsB) proteins revealed good conservation of residues within active sites (Figures 5.6 and 5.7). Multiple sequence alignment of the class II glycosyltransferase domains with *E. coli* otsA revealed complete conservation of 6 out of the 7 residues responsible for Glc6P binding, with one residue, arginine, replaced by alanine in all class II proteins (R9A; 9 depicting the residue number in the full otsA protein). Of the 9 residues responsible for UDP-Glc binding, 4 show complete conservation, 4 conservative replacements (i.e. replaced by an aa with similar biochemical properties; Q189H, R263D, F340V, M364L) and 1 radical replacement (i.e. replaced by an aa with different biochemical properties; G22D for PpTPS4 and PpTPS5). Analysis of *Arabidopsis* class II TPS proteins have shown that they have a similar degree of conservation of residues as the PpTPS class II proteins (data not shown). The exception lies in G22, which is deleted in *Arabidopsis* proteins, M364 which is conserved and E370K which represents a radical replacement.

Because the amino acids involved in substrate binding in the glycosyltransferase domain are mostly conserved, I decided to test whether these proteins possess TPS catalytic activity. Class II TPS proteins from vascular plants are catalytically inactive (Ramon et al., 2009; Zang et al., 2011), but this has not been examined in non-vascular plants. I complemented yeast mutants using plasmids expressing cDNA's encoding the proteins fused to HA tags (Figure 5.8). Yeast *tps1Δ* and t*ps1Δtps2Δ* mutants are unable to grow on glucose as a sole carbon source due to an uncontrolled influx of glucose into glycolysis resulting in ATP depletion. This is caused by hexokinase which becomes unregulated due to a lack or Tre6P in the mutant (Van Dijck et al., 2002). These mutants can, however, grow on galactose as the flux of this sugar into glycolysis is mediated by galactokinase which is not regulated by Tre6P. All constructs led to the synthesis of fusion proteins and all of the class II TPS proteins were unable to complement the *tps1Δ* and the *tps2Δ* mutants (Figure 5.8.A and C). However, one protein - PpTPS5 - led to growth on glucose in *tps1Δtps2Δ* double mutant revealing catalytic activity (Figure 5.8.B). A similar observation was made by Delorge et al. (2015), where two *Arabidopsis* class I TPS proteins (AtTPS2, AtTPS4) failed to complement the single mutant, but restored growth in the double mutant. The authors argued that complementation of the double mutant serves as a more sensitive test for TPS activity as TPP activity is also removed, which allows Tre6P to accumulate to sufficient concentrations that HXK activity becomes inhibited, allowing growth on glucose (Delorge et al., 2015). The ability of PpTPS5 to complement the *tps1Δtps2Δ* double mutant, therefore, indicates that the protein demonstrates TPS activity. As mentioned above, the residues responsible for Glc6P binding were highly conserved, while those responsible for UDP-Glc binding were not. The reason why only PpTPS5 exhibits activity is unclear as the substitutions in all four TPS2 proteins are very similar. PpTPS5 is most similar to PpTPS4, and the two proteins share 83.16% identity. Sites implicated in activity are replaced by the same residues in both proteins, indicating that novel sites must be present that affect activity which are altered in PpTPS4, but not on PpTPS5. It would be useful to determine whether class II proteins from other basal plant lineages also retain catalytic activity. If this could be demonstrated then comparison of protein sequences might reveal possible residues important for catalysis. This could be further substantiated using targeted mutagenesis to replace specific residues in non-catalytically active type II TPS isoforms.

5.4.4. Class II PpTPS proteins localize to the cytosol and do not interact with each other.

Previous studies have demonstrated cytosolic localization for TPS proteins. This has been demonstrated for AtTPS1, 2 and 4 via transient expression of GFP fusion constructs in *Arabidopsis* mesophyll protoplasts (Vandesteene et al., 2010). Furthermore, when AtTPS1 was expressed in transgenic tobacco immunogold labelling showed that it localized to the cytosol (Almeida et al., 2007). To study their subcellular localization, I expressed PpTPS-GFP fusions under the control of the constitutive 35S promoter and transiently expressed them in *P. patens* protoplasts prior to visualization by confocal microscopy and found that PpTPS3, PpTPS5 and PpTPS6 all localize to the cytosol (Figure 5.9). The localization of the active PpTPS5 isoform would allow it to synthesize Tre6P as that is where the substrates for its activity (Frc and UDP-Glc) are localised. The reasons for the cytosolic localization of the inactive class two proteins are, however, unclear. Researchers have proposed that class II proteins might be implicated in trehalose metabolism by acting as Tre6P sensors, allowing the plant to determine concentrations of this metabolite and bringing about responses to alterations (Ramon et al., 2009). It is also possible that class II TPS isoforms form complexes with other class I and II TPS proteins. Such complexes containing TPS proteins has been demonstrated in rice through co-immunoprecipitation and yeast two hybrid analyses (Zang et al., 2011). In that study, two class II proteins were found to interact with a class I protein (OsTPS1) and several other class II proteins. The authors hypothesized that the class II proteins may regulate the activity of OsTPS1 within a complex. To examine if the class II PpTPS proteins interact with each other and the class I proteins, I carried out a yeast two hybrid analysis where all of the TPS proteins (except PpTPS4) were tested as both bait and prey. None of the interactions tested resulted in growth (Figure 5.10), indicating that they do not. This indicates that the ability to form complexes evolved at a later point during evolution. However, a fuller picture needs to be obtained through examination both of the presence of complexes in basal land plants as well as examination using techniques other than the two-hybrid system. For example, purification TPS class II isoforms from plant crude extracts under native conditions and analysis of proteins that co-purify with them.

5.5. Conclusion

In summary I have identified and characterized four class II TPS genes in *P. patens*, using phylogenetic reconstruction, localization studies, yeast complementation and expression analyses. I have for the first time revealed catalytic activity in a class II protein, PpTPS5. Moreover, expression analyses indicated 1) that the genes are not functionally redundant and, 2) that certain duplicated genes may have acquired novel functions. A lack of catalytic activity in three of the class II genes might suggest that they may be indirectly involved in trehalose metabolism, either through the regulation of the catalytically active proteins or by monitoring Tre6P levels. Furthermore, the expression profile of PpTPS5 in response to heat stress, may indicate that it may be implicated in thermotolerance. Collectively, this has led to a greater understanding of the class II TPS genes in moss. Our future work will include studying class II proteins in other bryophytes to reveal whether activity has been retained in class II proteins in non-vascular plants.

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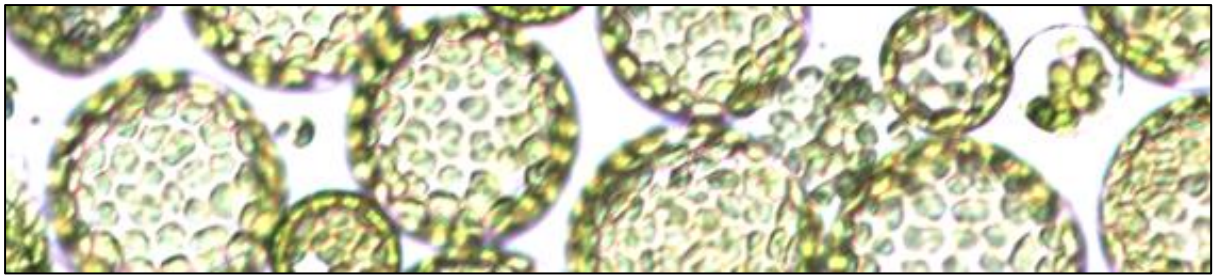

Chapter 6

General Discussion

As the end products of photosynthesis, sugars are central to carbon metabolism and, as an extension, vital to plant growth and development. Because of this they have acquired regulatory roles alongside their metabolic ones. These regulatory roles occur by sugars acting as signaling molecules that communicate information to the nucleus regarding the energy status of the plant, allowing it to adapt accordingly. The two sugars of interest in this study were sucrose and trehalose and plants are unique as they can synthesize both of these disaccharides. Both sugars are energy-rich and, because their constituent reducing monosaccharides are linked by their reducing ends, they are stable and non-reducing. However, plants accumulate different amounts of each. Sucrose accumulates in millimolar concentrations in most plants, while trehalose is normally present in only trace amounts. One exception to this is the presence of large amounts of trehalose in some resurrection plants. The reason for this difference may be attributed to one/some of the following reasons: (1) sucrose is more soluble, especially at low temperatures, making it more suited to being used in translocation; (2) sucrose degradation *via* SUS yields UDP-Glc, which is essential for cell wall synthesis, needed throughout development; (3) sucrose can be converted to fructans, which serve as storage carbohydrates; (4) high trehalose levels are incompatible with protein refolding during stress recovery and (5) some microbial pathogens are unable to metabolise sucrose, making it less readily available for them. Aside from the different levels to which these sugars accumulate, plants also encode different numbers of genes related to their metabolism. In *Arabidopsis*, 21 genes are putatively implicated in trehalose synthesis (e.g. 11 *TPS* and 10 *TPP*), compared to 8 for sucrose synthesis (e.g. 4 *SPS* and 4 *SPP*). Inversely, one gene is implicated in trehalose degradation (e.g. *TRE*), compared to 29 genes for sucrose degradation (e.g. 6 *SUS*, 13 *aINV*, 13 *nINV*). The variations in sugar levels, and the numbers of genes implicated in their metabolism, reflect the different biological functions for these two sugars and the Tre6P intermediate. Researchers are still

trying to unravel how the metabolism of these sugars are regulated in concert with the needs of the plant throughout growth and development. This study investigated two gene families implicated in sucrose and trehalose metabolism, *sucrose synthases* (*SUS*) and *trehalose 6-phosphate synthases* (*TPS*) respectively. These have been studied extensively in vascular plants, but not in non-vascular plants. I chose *P. patens* as a model bryophyte to carry out this study as it is a model plant with various protocols developed for its cultivation and has a sequenced genome, making the identification of putative homologs much easier. In this chapter I will briefly summarize the main findings of each experimental chapter and discuss directives for further experimentation that will strengthen some of the hypotheses put forward.

6.1. Characterization of the *P. patens SUS* multigene family and future directives.

Sucrose synthases (SUS) are glycosyl transferase enzymes implicated in sucrose metabolism predominantly in sink tissues, where they catalyse the reversible degradation of sucrose into Frc and UDP-Glc. These breakdown products are used in primary metabolism and the synthesis of complex carbohydrates. The size of *SUS* gene families vary considerably, with *Arabidopsis* encoding 6 (Baud et al., 2004) and *Pyrus bretschneideri* (pear) 30 (Abdullah et al., 2018). Plant *SUS* sequences normally differentiate into three clades. Our phylogenetic analysis revealed the existence of 5 clades, upon the inclusion of sequences from bryophytes and gymnosperms. Whether a sub-differentiation exists for bryophytes needs further investigation, for example by including more sequences from this class as they become available through genome sequencing. As it relates to angiosperm sequences, researchers have speculated that the differences between the clades may be coming from the expression profiles of the individual genes (Bieniawska et al., 2007). Our expression analyses indicated that three of the *P. patens* genes have similar profiles (*PpSUS2-4*) with one having a different profile (*PpSUS1*) over a day/night cycle. Expression in different tissues saw more variation. The different expression profiles over a day/night cycle might be reflected from their evolution, as *PpSUS1* appears to be the original *SUS* encoded by *P. patens*. The duplication events that followed may have led to neo-functionalisation of the new genes, whereby the duplicated genes evolved new functions. SUS proteins are primarily localized to the cytosol, but have been shown to be associated with plasma membranes, vacuoles, cell walls and mitochondria (Stein and Granot, 2019). Our localization studies revealed that all *Physcomitrella* SUS proteins were cytosolic. However, this does not exclude the possibility that they may bind to the plasma membrane for example, if targeted there after phosphorylation by a protein kinase. The creation of stable lines

expressing PpSUS-GFP fusion proteins and analysis of whole plant tissues (e.g. caulonemal or chloronemal filaments) would be able to shed more light in this regard. Using constructs that harbour multiple cassettes, co-localization studies can be conducted within the same stable line, using markers fused to different fluorophores. If, however, they are strictly cytosolic, it would mean that the dynamic localization of SUS proteins in vascular plants may have evolved as an adaptation to life away from water, where protein versatility is required.

Future studies need to include kinetic characterization of the PpSUS proteins to reveal which proteins are catalytically active. This can be followed up by the generation of knock-out lines to determine which of the homologs are important to the plant, or, whether they have overlapping function. Because *PpSUS* genes have distinct expression patterns as it relates to development, I can hypothesize that they play specific roles. It would be interesting to induce sporophyte development to reveal how this is affected in a *ppsus3* mutant. A cucumber (*Cucumis sativus L.*) transgenic line with downregulated *CsSUS4* expression, had flowers and fruits reduced in size and weight, with reduced hexose, starch and cellulose levels (Fan et al., 2019). Conversely, *CsSUS4* overexpression lines showed the inverse phenotype. Similarly, a *ppsus1/ppsus2* double knock-out could reveal the role SUS plays in female gametophyte (archegonia) development. Clearly a quadruple knock-out would need to be generated to ascertain the importance of SUS overall in *P. patens*, or whether INV can compensate for their loss, as has been shown in *Arabidopsis* (Baroja-Fernández et al., 2012). Promoter-GUS fusions can also be used to strengthen *in silico* expression data to reveal in which tissues and under which conditions expression is highest to help identify potential roles.

Knock-out lines can also be used to test whether PpSUS proteins participate in photosynthate translocation in both the gametophyte and sporophyte. Single and double knock-outs of *PpSUS1* and *PpSUS2* could reveal whether SUS is needed to establish sink cells/tissues, by looking at whether colony expansion and bud formation is affected. Similarly, knock-outs of *PpSUS1*, *PpSUS2* and *PpSUS3* could be used to test the model put forth by Regmi et al., (2017) as it relates to photosynthate translocation to the nutrionally dependent sporophyte.

6.2. Characterization of the *P. patens* class II *TPS* genes and future directives.

All life forms contain trehalose and possess enzymes that degrade it to glucose. Plants have large *TPS* and *TPP* gene families, which are ancient in origin, dating back to before the streptophyte and chlorophyte divergence. These gene families are amplified in plants, with

Arabidopsis encoding 11 *TPS* and 10 *TPP* genes. A eukaryotic origin has been proposed for *TPS* genes, since they are more closely related to fungi and other eukaryotes, whereas a prokaryotic origin was proposed for *TPP* genes, which are closely related to bacteria. Trehalose 6-phosphate and TPSs, have received much attention over the past quarter century. Most research has been conducted on the class I *TPS* genes with very little attention given to class II genes, as they encode catalytically inactive enzymes. Furthermore, no studies have been carried out on *TPS* genes in non-vascular plants. In chapter 5 I sought, therefore, to simultaneously address these two shortcomings in the field by characterizing the class II *TPS* homologs in *P. patens.*

It has been proposed that the acquisition of bacterial *TPP* genes drove the evolution of new functions for the class II TPS proteins, since they lack both TPS and TPP activity (Lunn, 2007). Here the author proposed that class II proteins possessed TPP activity prior to the acquisition of bacterial *TPP* genes, due to the similarity in their domain structures to yeast ScTPS2 (TPP activity; Lunn, 2007). As a consequence, their catalytic activity may have become redundant over time, leading the genes encoding them, to evolve new functions. In this study I have demonstrated that one class II protein, PpTPS5, still retains catalytic activity, suggesting that it is an ancestral class II protein that has retained its original functionality, unlike the other class II proteins that have potentially acquired novel, perhaps regulatory, functions. This finding has important implications about the evolution of TPS isoforms. Future experimentation firstly needs to include an assay to measure Tre6P liberation to confirm that PpTPS5 is catalytically active. Furthermore, class II proteins will need to be studied in other bryophytes as well as red, brown and green algae to determine whether other non-vascular plants retained functionality in some of their class II proteins. In addition, the identification of an active class II TPS will help in identifying amino acid residues important for activity. One of the functions proposed for catalytically inactive class II proteins is that they act as regulatory subunits of a TPS complex. While it may be true that class II proteins form part of complexes in certain vascular plants, this capability probably evolved later, since it was not observed in *P. patens.* A second possible function for class II proteins is that they may act as Tre6P sensors, however, whether this is the case for *P. patens* is unclear. In vascular plants, class II proteins are subject to a high degree of regulation, especially at the transcriptional level. The four class II *PpTPS* genes show similar expression profiles over a day/night cycle and in different tissues, however, differential expression was observed when plants were subjected to hormone and heat stress treatments. This could reflect distinct roles for the individual gene products.

Future experiments need to include the generation of knock-out lines to determine if class II proteins are important to *P. patens*, or whether they can compensate for one another. Quadruple knockouts should also be generated to determine whether the class I proteins can compensate for a loss in the class II proteins. Promoter-GUS fusions can also be used to strengthen *in silico* expression data to reveal in which tissues and under which conditions expression is highest.

6.3. Closing remarks

In conclusion, this project aimed to characterize two gene families involved in disaccharide metabolism and signaling in *P. patens*. Novel insights have been gained for both gene families. I have found that *PpSUS* sequences occupy a novel bryophyte-specific clade among *SUS* sequences from land plants and, have for the first time demonstrated catalytic activity for a class II TPS protein. Future work into both gene families will shed further light on sucrose and trehalose metabolism and signaling within non-vascular plants.

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